Aditya Pratap · Jitendra Kumar *Editors*

Alien Gene Transfer in Crop Plants, Volume 1

Innovations, Methods and Risk Assessment

Alien Gene Transfer in Crop Plants, Volume 1

Aditya Pratap • Jitendra Kumar
Editors

Alien Gene Transfer in Crop Plants, Volume 1

Innovations, Methods and Risk Assessment

Editors
Aditya Pratap
Senior Scientist (Plant Breeding)
Crop Improvement Division
Indian Institute of Pulses Research
Kanpur, India

Jitendra Kumar
Senior Scientist (Plant Breeding)
Crop Improvement Division
Indian Institute of Pulses Research
Kanpur, India

ISBN 978-1-4614-8584-1 ISBN 978-1-4614-8585-8 (eBook)
DOI 10.1007/978-1-4614-8585-8
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2013950179

© Springer Science+Business Media New York 2014
This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed. Exempted from this legal reservation are brief excerpts in connection with reviews or scholarly analysis or material supplied specifically for the purpose of being entered and executed on a computer system, for exclusive use by the purchaser of the work. Duplication of this publication or parts thereof is permitted only under the provisions of the Copyright Law of the Publisher's location, in its current version, and permission for use must always be obtained from Springer. Permissions for use may be obtained through RightsLink at the Copyright Clearance Center. Violations are liable to prosecution under the respective Copyright Law.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
While the advice and information in this book are believed to be true and accurate at the date of publication, neither the authors nor the editors nor the publisher can accept any legal responsibility for any errors or omissions that may be made. The publisher makes no warranty, express or implied, with respect to the material contained herein.

Printed on acid-free paper

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

Foreword

Plant breeding as a science began at the advent of nineteenth century with the discoveries on inheritance of plant traits. Travelling through natural transfer and reassortment of chromatin within heterogeneous cultivated populations and later through deliberate gene transfers within the species, it has reached to the stage of current day precise, accelerated and target-oriented vertical and horizontal alien gene transfers. The quest of researchers for newer sources of genetic variation led the crop development programmes to look for genes of interest not only in the secondary and tertiary gene pools but also even beyond the genome boundaries. Nevertheless, the evolution of alien gene transfer technology to its present form has taken considerable time owing to several factors, primarily the scepticism of plant breeders toward use of wild and exotic plant genetic resources due to associated linkage drag, variable expression of alien genes in cultivated backgrounds, pre- and post-fertilization barriers, recalcitrancy of some of the crop species for in vitro techniques and non-availability of genes of interest within the gene pool. Manipulations in hybridization procedures and in vitro techniques and support by modern tools including molecular marker technology, molecular cytogenetics, genetic transformation and bioinformatics have, however, provided solutions to many of the above problems and developed confidence among plant breeders to take up alien gene

transfer into crop plants as a routine practice. Consequently, the gains from such transfers have been many and examples of successful alien gene transfers are scattered across a wide range of crop plants and large number of traits *viz*., resistance to biotic and abiotic stresses, improved nutrition, increased keeping quality, non-shattering and sometimes easy-shattering habit, and an overall enhanced economic recovery as compared to the earlier types. Albeit, the alien gene transfers, particularly horizontal gene transfer, have also raised some concerns as to the long-term ecological and social impacts which require to be addressed with more scientific reasoning to convince the end users about their safety and economic viability.

This book, "Alien gene transfer in crop plants: Innovations, methods and risk assessment" compiled by two young scientists of this institute provides an insight into various spheres of this important aspect. The editors have meticulously covered the methods, newer innovations, detection, challenges and risks associated with alien gene transfer. Voluminous literature is available on development and use of alien introgressions in crop plants, and it would have been definitely an uphill task for the editors to consolidate the most significant aspects on alien gene transfer in a single book. For their sincere efforts, they deserve appreciations. All the chapters in this book are contributed by renowned scientists whose contributions are well recognized. I am sure that the information contained herein will motivate plant scientists to use wild and distant plant genetic resources in improving the crop plants further and take alien gene transfer as a routine practice wherever obligatory, and also that it will be a useful knowledge base for those involved in teaching and research of agriculture and allied subjects.

Kanpur, India N. Nadarajan

Preface

Transfer of alien genes into crop plants from wild and exotic plant genetic resources has invoked tremendous interest of crop scientists globally. Wild species are a rich reservoir of useful alien genes hitherto not available in the cultivated gene pool. These include resistance to diseases and insect-pests, tolerance to drought, salinity, temperature extremities and other abiotic stresses as well as genes for several quality traits. While most of the alien gene introgressions of practical importance in crop plants have been achieved through vertical gene transfer, horizontal gene transfer through transgenesis (for cross incompatible species), somatic hybridization and most recently, intragenesis and cisgenesis, has offered great promise in broadening the genetic base of cultivated crop species. These techniques, lately aided by molecular markers and in situ hybridization have led to introgression of hundreds of genes of interest in cultivated background, thereby improving their genetic potential. The gains through alien gene transfer are significant, nevertheless also raising some issues regarding their possible impacts on human and animal health as well as on environment. Even though, such gene transfers have been successfully accomplished across many crop species and technologies required for these transfers have been refined, which significantly improved the success rate of alien introgression events. Consequently, besides development of several plant products, ample literature has been generated over the years on different aspects of alien gene transfer which needed to be brought under a single book cover so as to provide the readers a comprehensive exposition on this important aspect. Realizing it, we developed this theme with an objective to provide an overview about the importance of alien gene transfer, how it is accomplished, detection of introgressions, the associated advantages and risks, and the significant achievements made from alien gene transfers. Keeping in view the scope of the subject, we have covered this topic in two volumes; the first volume deals with the innovations, methods and risk assessment while the second volume deals more with the practical aspects and covers achievements and impacts of alien gene transfer.

The first volume is already in your hands and covers more of the theoretical aspects of alien gene transfer in crop plants. The first chapter introduces the topic and discusses various techniques of alien introgression followed by a chapter on

distant hybridization, which is a prerequisite for vertical gene transfer. The subsequent two chapters deal with the important aspects of tissue culture and embryo rescue followed by a chapter on techniques of horizontal gene transfer through genetic transformation. Distant hybridization has also led to the discovery of newer techniques immensely useful to plants breeders, and Chap. 6 explains one such technique—doubled haploidy breeding. The following two chapters cover the modern aspects of molecular techniques helping in introgression as well as detection of alien chromatin in cultivated background. Chapter 9 elaborates some of the significant agronomically relevant traits transferred into crop plants. Of late, bioinformatics has witnessed tremendous developments and finds great uses in many spheres of agricultural research, including the detection of alien genes, and this aspect has been covered in Chap. 10. The subsequent two chapters summarize the theme specially focussing on the possible human and ecological impacts of alien gene transfers as well the challenges and risks involved.

The authors of various chapters of this book are all renowned experts in their fields and deserve heartfelt thanks for writing their chapters meticulously and with great responsibility. We are extremely thankful to Dr. S. Ayyappan, Secretary, Department Agricultural Research and Education, Government of India and Director General, Indian Council of Agricultural Research (ICAR), New Delhi, for providing overall support and guidance in furthering our research and academic pursuits. Prof. Swapan Kumar Datta, Deputy Director General (Crop Science), ICAR and Dr. B.B. Singh, Additional Director General (Oilseeds and Pulses), ICAR deserve special mention for their constant encouragement for taking up this endeavour. With profound gratitude we also wish to mention the name of Dr. N. Nadarajan, himself an accomplished plant breeder and Director, Indian Institute of Pulses Research Kanpur, who has a special interest in the subject of distant hybridization and alien gene transfer in crop plants. He was a key force in motivating us to undertake this endeavour and deserves our appreciations. We are also grateful to our colleague Debjyoti Sen Gupta and research scholars working with us: Nupur Malviya, Rakhi Tomar, Ekta Srivastava and Mrityunjaya Singh for compilation of references and searching voluminous literature related to the topic. At Springer, Hannah Smith, Mellissa Higgs and Kenneth Teng, the commissioning editors; Daniel Dominguez the developmental editor and the entire production team have been instrumental in developing our idea of a book on such an important subject to its present form and deserve our appreciations. Our lovely kids Puranjay, Neha and Gun always helped to keep the atmosphere lively while Dr. Rakhi Gupta and Mrs. Renu Rani, our better halves, allowed us to work overtime and gave us all emotional support for which they deserve our genuine appreciation.

We hope that this book will be successful in achieving what we actually desired from it—providing the readers an updated and comprehensive reference on alien gene transfer in crop plants and a ready reckoner for the researchers and scholars who have an interest in this field.

Kanpur, UP, India

Aditya Pratap
Jitendra Kumar

Contents

1 **Alien Gene Transfer in Crop Plants: An Introduction** 1
Aditya Pratap and Jitendra Kumar

2 **Distant Hybridization: A Tool for Interspecific Manipulation of Chromosomes** 25
Dengcai Liu, Huaigang Zhang, Lianquan Zhang, Zhongwei Yuan, Ming Hao, and Youliang Zheng

3 **Tissue Culture and Regeneration: A Prerequisite for Alien Gene Transfer** 43
Maria Wędzony, Magdalena Szechyńska-Hebda, Iwona Żur, Ewa Dubas, and Monika Krzewska

4 **Methods and Role of Embryo Rescue Technique in Alien Gene Transfer** 77
Monika M. Lulsdorf, Alison Ferrie, Susan M.H. Slater, and Hai Ying Yuan

5 **Horizontal Gene Transfer Through Genetic Transformation** 105
Pooja Bhatnagar-Mathur, Paramita Palit, and K.K. Sharma

6 **Distant Hybridisation and Doubled-Haploidy Breeding** 143
Harinder K. Chaudhary, Vineeta Kaila, Shoukat A. Rather, and Tisu Tayeng

7 **Role of Molecular Markers** 165
Reyazul Rouf Mir, Javaid Akhter Bhat, Nelofer Jan, Bikram Singh, Ashok Kumar Razdan, Mohd Ashraf Bhat, Ajay Kumar, Ekta Srivastava, and Nupur Malviya

8 **Molecular Cytogenetics for Identification of Alien Chromosomes and Chromosome Segments** 187
Harinder K. Chaudhary, Vineeta Kaila, and Shoukat Ahmad Rather

9 **Agronomically Relevant Traits Transferred to Major Crop Plants by Alien Introgressions** 211
Neeraj Kumar and Sachin Rustgi

10 **Gene Flow and Risk Assessment in Genetically Modified Crops** 247
Stephen F. Chandler and Trevor W. Stevenson

11 **Bioinformatics Approaches to Deciphering Alien Gene Transfer: A Comprehensive Analysis** 267
Rajeev K. Azad, Nitish Mishra, Firoz Ahmed, and Rakesh Kaundal

12 **Alien Gene Transfer: Challenges and Opportunities** 289
Jitendra Kumar and Aditya Pratap

About the Editors 309

Index 311

Contributors

Firoz Ahmed The Samuel Roberts Noble Foundation, Ardmore, OK, USA

Rajeev K. Azad Departments of Biological Sciences and Mathematics, University of North Texas, Denton, TX, USA

Javaid Akhter Bhat Division of Plant Breeding & Genetics, Shere-Kashmir University of Agricultural Sciences & Technology of Jammu, Jammu, India

Mohd Ashraf Bhat Molecular Biology Laboratory, Division of Plant Breeding & Genetics, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir, Kashmir, India

Pooja Bhatnagar-Mathur Genetic Transformation Laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India

Stephen F. Chandler School of Applied Sciences, RMIT University, Melbourne, VIC, Australia

Harinder K. Chaudhary Molecular Cytogenetics and Tissue Culture Laboratory, Department of Crop Improvement, CSK HP Agricultural University, Palampur, India

Ewa Dubas Institute of Plant Physiology, Polish Academy of Sciences, Krakow, Poland

Alison Ferrie National Research Council of Canada (NRCC), Saskatoon, SK, Canada

Min Hao Triticeae Research Institute, Sichuan Agricultural University, Wenjiang, Sichuan, P.R. China

Nelofer Jan Department of Botany, Chaudhary Charan Singh University, Meerut, India

Vineeta Kaila Molecular Cytogenetics and Tissue Culture Laboratory, Department of Crop Improvement, CSK HP Agricultural University, Palampur, India

Rakesh Kaundal Department of Biochemistry & Molecular Biology, National Institute for Microbial Forensics & Food and Agricultural Biosecurity (NIMFFAB), Oklahoma State University, Stillwater, OK, USA

Monika Krzewska Institute of Plant Physiology, Polish Academy of Sciences, Krakow, Poland

Ajay Kumar Department of Plant Sciences, North Dakota State University, Fargo, ND, USA

Jitendra Kumar Crop Improvement Division, Indian Institute of Pulses Research, Kanpur, Uttar Pradesh, India

Neeraj Kumar Department of Crop and Soil Sciences, Washington State University, Pullman, WA, USA

Dengcai Liu Key Laboratory of Adaptation and Evolution of Plateau Biota, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining, P.R. China

Triticeae Research Institute, Sichuan Agricultural University, Wenjiang, Sichuan, P.R. China

Monika M. Lulsdorf Crop Development Centre (CDC), University of Saskatchewan, Saskatoon, SK, Canada

Nupur Malviya Division of Crop Improvement, Indian Institute of Pulses Research, Kanpur, India

Reyazul Rouf Mir Division of Plant Breeding & Genetics, Shere-Kashmir University of Agricultural Sciences & Technology of Jammu, Jammu, India

Nitish Mishra The Samuel Roberts Noble Foundation, Ardmore, OK, USA

Paramita Palit Genetic Transformation Laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India

Aditya Pratap Crop Improvement Division, Indian Institute of Pulses Research, Kanpur, Uttar Pradesh, India

Shoukat A. Rather Molecular Cytogenetics and Tissue Culture Laboratory, Department of Crop Improvement, CSK HP Agricultural University, Palampur, India

Ashok Kumar Razdan Division of Plant Breeding & Genetics, Shere-Kashmir University of Agricultural Sciences & Technology of Jammu, Jammu, India

Sachin Rustgi Department of Crop and Soil Sciences, Washington State University, Pullman, WA, USA

K.K. Sharma Genetic Transformation Laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India

Bikram Singh Division of Plant Breeding & Genetics, Shere-Kashmir University of Agricultural Sciences & Technology of Jammu, Jammu, India

Susan M.H. Slater Crop Development Centre (CDC), University of Saskatchewan, Saskatoon, SK, Canada

Ekta Srivastava Division of Crop Improvement, Indian Institute of Pulses Research, Kanpur, India

Trevor W. Stevenson School of Applied Sciences, RMIT University, Melbourne, VIC, Australia

Magdalena Szechyńska-Hebda Institute of Plant Physiology, Polish Academy of Sciences, Krakow, Poland

Tisu Tayeng Molecular Cytogenetics & Tissue Culture Laboratory, Department of Crop Improvement, CSK Himachal Pradesh Agricultural University, Palampur, India

Maria Wędzony Pedagogical University of Krakow, Krakow, Poland

Hai Ying Yuan Crop Development Centre (CDC), University of Saskatchewan, Saskatoon, SK, Canada

Zhongwei Yuan Triticeae Research Institute, Sichuan Agricultural University, Wenjiang, Sichuan, P.R. China

Huaigang Zhang Key Laboratory of Adaptation and Evolution of Plateau Biota, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining, P.R. China

Lianquan Zhang Triticeae Research Institute, Sichuan Agricultural University, Wenjiang, Sichuan, P.R. China

Youliang Zheng Triticeae Research Institute, Sichuan Agricultural University, Wenjiang, Sichuan, P.R. China

Iwona Żur Institute of Plant Physiology, Polish Academy of Sciences, Krakow, Poland

Chapter 1
Alien Gene Transfer in Crop Plants: An Introduction

Aditya Pratap and Jitendra Kumar

Abstract Alien gene transfer in crop plants has led to tremendous improvement in various crop species. Wild species are rich resources of useful alien genes which are not available in the cultivated gene pool. These include genes for resistance to diseases and insect pests; for tolerance to drought, salinity, temperature extremities and other abiotic stresses as well as for quality traits. While most of the alien gene introgressions in crop plants have been achieved through vertical gene transfer, horizontal gene transfer through transgenesis, somatic hybridization and, most recently, intragenesis and cisgenesis has invoked tremendous interest of the scientific community globally. These techniques, lately aided by molecular markers and in situ hybridization, together have led to introgression of hundreds of genes of interest in cultivated background of crop species, thereby improving their genetic potential. This chapter provides an insight into importance and need of alien gene transfer, various methods to achieve it, alien gene detection and role of alien gene transfer in creating variability.

Keywords Distant hybridization • Cisgenesis • Crop evolution • Gene flow • Genetic transformation • Intragenesis • Introgression • Reproductive barriers • Somatic hybridization

---

A. Pratap, Ph.D. (✉) • J. Kumar
Crop Improvement Division, Indian Institute of Pulses Research,
Kanpur, Uttar Pradesh 208 024, India
e-mail: adityapratapgarg@gmail.com

A. Pratap and J. Kumar (eds.), *Alien Gene Transfer in Crop Plants, Volume 1: Innovations, Methods and Risk Assessment*, DOI 10.1007/978-1-4614-8585-8_1,
© Springer Science+Business Media New York 2014

1.1 Introduction

The twentieth century has witnessed tremendous improvement in global crop production. Besides several factors such as increase in cultivated area, improved agronomic practices, increased use of plant protection measures and better crop management, improved varieties of crop plants have played a dramatic role in improving the productivity of different crops. The genetically improved crop cultivars have been developed through modern plant breeding by introducing improved alleles at existing loci through conventional hybridization, of late, aided by molecular marker technology and genetic transformation. The aim of all these techniques has been either exchange of genes between sympatric or neighbouring populations of crops and related taxa or transfer of genes from related taxa into the cultivated gene pool of a crop. This led to the development of numerous improved cultivars with high yield, stress resistance and superior agronomic performance. In nature, gene transfer from one population to another is slow as compared to man-made systems, whereas it is faster and often mediated by hybridization followed by a number of backcrossings and rigorous phenotypic selections.

Hybridization has been known to occur at least since the times of Linnaeus and discussed by several evolutionists (Mayr 1963; Coyne and Orr 2004). The introduction of foreign genes into the gene pool of a crop species by human intervention has been used by plant breeders and applied geneticists for almost 100 years. Natural selection and, of late, human selection have helped in maintaining new combinations of genes, and these gene combinations have been transferred through hybridization between cultivated and wild taxa leading to the development of populations with new characteristics (i.e. increased genetic diversity of modern crops) (Anderson 1949; Arnold 1992) and evolution of domestic crop species (Stebbins 1959; Slatkin 1987; Jarvis and Hodgkin 1999). Breeders and geneticists have increasingly sought new sources of resistance in diverse germplasm, often involving distant and wild relatives (Gill et al. 2011). While there are several instances of deliberate introgression of desirable traits into crop cultivars as a part of regular plant breeding programmes, the extent and impact of farmer-aided or natural introgression are uncertain (Jarvis and Hodgkin 1998; 1999).

During the crop domestication, few species of crops were selected for cultivation. As a result narrow germplasm forms the basis of modern monoculture in many areas of the world (Gill et al. 2011). Therefore, use of vertical transfer for alien gene(s) could be restricted mainly to crossable wild species. Nevertheless, horizontal transfer (HGT) of alien genes from non-crossable wild species or even across the genera is now increasingly being recognized as a significant and potent force in the evolution of eukaryotic genomes (Bock 2009). While somatic hybridization after the initial leaps slowed down in yielding practical outputs, the ability to transform crop plants has developed remarkably since the first genetically transformed plants were reported in 1983. Transgenics have a potential to significantly increase the genetic component of integrated pest management through the development

of insect-resistant cultivars with very strong in-built insecticidal properties, comparable to those of chemical pesticides (Pratap and Gupta 2009a). The use of transgenics for crop protection from insects, disease and weeds is further expected to increase tremendously, while ethical and environmental concerns regarding the development and use of transgenic crop varieties may be addressed by latest introduction of technologies like intragenesis and cisgenesis. This chapter provides an overview on alien gene transfer in crop plants and its implications in creating variability and evolution of crop species.

1.2 Need for Alien Gene Transfer

Genetic variation is essential for developing new plant varieties, and this can be created by introducing genes from a related species, sometimes from a relatively distant species or even an unrelated species. The need for gene transfer in a crop species depends upon the extant genetic variability in that crop as well as availability of a trait of interest in the donor in intense form. However, the valuable genes available within a crop species are easier to manipulate as compared to the alien genes, especially in the case of quantitative traits. Gene transfer within a species is not usually associated with undesirable effects which is one of the major limitations in alien gene transfer from distant species due to linkage of desirable genes with undesirable ones (linkage drag). In most of the cultivated crop species, limited popular and high-yielding varieties are grown over wide areas and these are often derived from a relatively narrow representation of gene pool, mostly from the primary gene pool, and therefore these have a narrow genetic base and limited genetic buffer. In this way, modern plant breeding although increased crop productivity worldwide, it also eroded the genetic variability of the crops (Hoisington et al. 1999). Consequently, our major crop species represent the relatively few species that were selected by our ancestors from a multitude of extant species, and the resulting narrow germplasm forms the basis of modern monoculture in many areas of the world (Gill et al. 2011). This makes them fragile to global climate change and vulnerable to new races of pathogens and insect pests. Due to narrow genetic variability, options to execute selection for desirable plant types also become limited. Since plant breeding in practice offers an option for crop improvement, efforts have been made to search for genes imparting resistance to stresses within the cultivated species and to a limited extent among their wild relatives, but success has been limited to a few diseases and insect pests that are confined to major gene(s) from the primary gene pool in most of the crop plants (Knott and Dvorak 1976; Stalker 1980; Ladizinsky et al. 1988; Prescott-Allen and Prescott-Allen 1986, 1988; Hajjar and Hodgkin 2007; Kumar et al. 2011a, b). To diversify and broaden the genetic base of cultivated germplasm, introgression of alien genes from wild species offers a viable option not only to minimize the risk of stress epidemics but also to make discernible yield advances in crop species.

Wild species are a rich reservoir of useful alien genes which are no longer available within the cultivated gene pool (Tanksley and McCouch 1997; Pratap and Gupta 2009b). Since these species have had much longer time and increased opportunities to evolve and adapt to natural environments, therefore, these often have genes for resistance to diseases and insect pests and for tolerance to drought, temperature stress, salinity and other extreme environmental conditions. Further, they have wide genetic buffers to withstand unexpected adversities. Therefore, gene transfer from distant and wild genetic resources provides an opportunity for development of additional variability as well as incorporation of desirable genes hitherto not available in cultivated germplasm. Natural introgressions between wild relatives and their crop cultivars continue to be a factor in increasing the genetic diversity of modern crops today (Anderson 1949, 1961; Arnold 1992; Altieri and Montecinos 1993).

1.3 Methods of Alien Gene Transfer

There are two ways to transfer the alien gene(s) into cultivated species: transferring alien gene from cross-compatible wild species through hybridization (vertical gene transfer) and transfer of gene(s) from other sources as well as cross-incompatible wild species through genetic transformation and somatic hybridization (horizontal gene transfer) (Fig. 1.1). These methods of gene transfer have been discussed briefly below and elsewhere in this book.

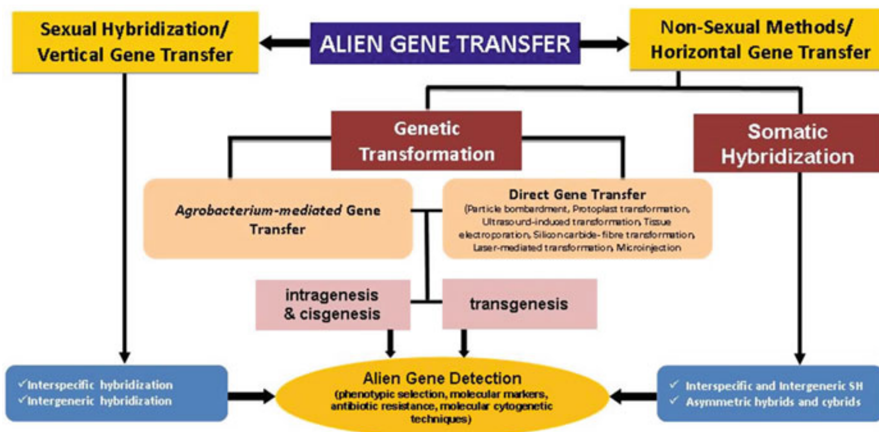

Fig. 1.1 Methods of alien gene transfer in crop plants

1.3.1 Vertical Gene Transfer

Gene transfer in nature occurs by the movement of genes across different populations, conferring new traits to the individuals in the recipient population. Thus during this process the genetic information is transferred from parents to offspring, which is known as vertical gene transfer (VGT). Therefore, it is only possible in those cases where the individuals can mate with each other naturally or by artificial means and consequently are able to produce the offspring. VGT is usually involved in plant breeding where plants with desired traits are selected after the sexual transfer of genes by cross-fertilization between two parents (Goodman et al. 1987). In case of one of the parents being a cultivated variety and the other being a wild relative, gene transfer is usually accomplished by several rounds of backcrossing with the cultivated parent and stringent selection for the desirable recombinant.

VGT occurs most frequently and successfully within the primary gene pool of a crop species as classified by Harlan and de Wet (1971). Gene transfer can occur spontaneously only when the crop cultivation and the distribution of its relatives are more or less sympatric or at least overlapping locally, and they are cross compatible and produce somewhat fertile offspring (Hanelt 1997). Crops which have been separated through true speciation or reproductive isolation cannot have gene transfer between them through hybridization.

Plant breeding is selection of plants with desired traits after the sexual transfer of genes by cross-fertilization between two parents (Goodman et al. 1987). Systematic plant breeding began in the nineteenth century with the studies that how plant traits are inherited. The early years witnessed transfer and reassortment of a large number of genes in heterogeneous cultivated populations which was followed by breeders expanding their search for new genetic variation to the entire crop species, including non-cultivated populations (Goodman et al. 1987). However, the earlier gene transfer events focused mainly within the species. This was followed by the search of plant breeders for newer variability which led to exploration of interspecific and intergeneric gene transfer methods later on and their use in improving cultivated crops. Gene transfer from related species into cultivated wheat began in 1930 when McFadden (1930) transferred resistance to stem rust and loose smut diseases from tetraploid *Triticum tauschii* to hexaploid *T. aestivum*. This led to the development of rust-resistant variety “Hope” which was cultivated in the United States for a very long time. Later in 1936, Tucker and Bohn transferred a gene conferring resistance to race 1 of *Fusarium* wilt fungus from a weedy plant, *Lycopersicon pimpinellifolium*, to cultivated tomato, *L. esculentum*.

In the twentieth century, plant breeders had increasingly used interspecific hybridization for the transfer of genes from a non-cultivated plant species to a variety in a related crop species. One of the classical examples of such gene transfer is that of transfer of leaf rust resistance from *Aegilops umbellulata* and yellow rust

resistance from *A. comosa* into cultivated wheat (*Triticum aestivum*) (Sears 1956; Riley et al. 1968). This was accomplished through hybridizations and chromosome manipulations. Another classical example is that of breeding new tomato cultivars with increased dry matter content using small wild Peruvian tomato, *Lycopersicon chmielewskii*, which has high soluble solid contents (Rick 1974; Iltis 1988). This was also accomplished by hybridization, backcrossing and pedigree selection. Similarly, genes for resistance to races of stem rust and powdery mildew and also Hessian fly have been incorporated from *T. tomopheevi*, *T. monococcum* and *T. turgidum* in many bread wheat varieties (Goodman et al. 1987). Later some more useful genes from wild relatives of crop plants have been incorporated into cultivated background of cereals (Jauhar 1993; Jiang et al. 1994; Jauhar and Chibbar 1999), oilseeds, food legumes (Kumar et al. 2011b) and other crops. There are now hundreds of reports of alien gene transfer over several agricultural crops which will be discussed in subsequent chapters of this volume as well as in different crop-specific chapters in Volume II.

1.3.2 Factors Affecting Alien Gene Transfer Through Hybridization

Hybridization is a frequent and important component of plant evolution and speciation (Riesberg and Ellstrnd 1993). The value of hybridization in transfer of alien genes into cultivated background has been known for a long time. Chromosome pairing between chromosomes of the alien donor and those of the cultivated crops is the key to such gene transfers.

For successful gene transfer through hybridization, cross-pollination must occur and there should not be any pre-mating and post-mating barriers. Prerequisites for hybridization include sympatric parents that occupy similar habitats, overlapping flowering times, similar pollinators and intertaxa compatibility (Darwin 1859; Sarr et al. 1988). Hybridization and subsequent gene transfer depend upon several biological and environmental conditions. These include the following: sexually compatible species must be growing within a pollen or a seed dispersal range of the crop, the phenology (flowering and fertilization) of the donor and recipient population must overlap, fertile and viable offspring must be produced as a result of hybridization between two species and the offspring of the hybrid plant must be fertile and viable. The above conditions are tremendously affected by the biology of donor and recipient plants as well as prevailing ecological conditions. Even when these conditions are fulfilled and gene transfer is likely, the advantages of gene transfer may reverse or reduce over time while in some cases they may even lead to improved vigour (Hauser et al. 1998). Nevertheless, there must be a minimum level of fertility in the offspring of the recipient population so that the transgene is maintained and passed on to next generation. In such cases if the resulting embryos develop into viable seeds and germinate, the F_1 plants typically have some reduced fertility but are rarely fully sterile.

The low frequency of hybrids between most species, on a per individual basis, is largely explained by pre- and post-zygotic barriers (Mallet 2005). Further, incompatibilities between populations are strongly affected by selection and so are not expected to evolve in a regular or a time-bound manner. The taxon-specific differences may also account for increased hybridization rates within some taxa while less or rare hybridization and consequently negligible gene transfer in the other. Gene transfer can occur at different taxonomic levels. Accordingly, gene transfer can be classified as intraspecific, interspecific and intergeneric. However, the extent of gene transfer is influenced by the objective of transfer, trait under transfer, breeding system of the partners involved, availability of pollinating agents, layout of the trial (under experimental conditions) and population size.

For the success of gene transfers between cultivated and wild species, chromosome pairing between chromosomes of the alien donor and cultivated species is most important as the success of gene transfer from donor to the recipient species depends upon the degree of chromosome homology that exists between these two species. Relatively easier gene transfers occur between diploid species such as maize and barley where only one genome needs to be constructed. For example, *Hordeum bulbosum* and *H. spontaneum* are useful sources for improvement of barley (Repellin et al. 2001).

1.3.3 Reproductive Barriers

Reproductive barriers limit alien gene transfer through interspecific and intergeneric hybridization. The sexual barriers hampering distant hybridization have been distinguished into pre- and post-fertilization barriers (Stebbins 1958). These include those barriers that reduce the chances of formation of a viable zygote (pre-zygotic barriers) and those which are due to lower survival or reproductive fitness of the hybrids (post-zygotic barriers). The pre-zygotic barriers include ecological or habitat isolation, temporal differences in flowering phenology or pollinator service, temporal separation in flowering and/or pollination time between a pair of closely related species and gametophytic isolation. Gametophytic isolation is a pre-zygotic post-pollination mechanism which prevents fertilization by the pollen of foreign species.

Post-zygotic barriers may occur at various developmental stages of the hybrid progeny. The seeds may sometimes fail to develop due to degeneration of endosperm of the hybrid seed leading to scarcity of nutrition to the developing seed. Even the plants may develop from the hybrid seeds but they may die before they are able to reproduce. Sometimes mature hybrid individuals may be sterile, the sterility being manifested in various stages of the reproductive cycle, for example failure to produce viable pollens or ovules, abortion of the embryo or unviable seeds (Futuyma 1998).

The use of mixed pollen, i.e. mixture of compatible and incompatible pollen (Brown and Adiwilaga 1991) and mentor pollen, i.e. compatible pollen genetically

inactivated by irradiation but still capable of germination, is reported to overcome inhibition in the style in many plant species. As it was first demonstrated more than 60 years ago in *Datura* (Blakeslee 1945), pollen tube growth inhibition in the style can be overcome using different pollination techniques in which style and ovary are manipulated. One of these manipulations involves removing the stigma and a part or whole of the style and pollinating the cut end. Application of growth regulators, such as auxins, cytokinins and gibberellins, to the pedicel or the ovary at the time of or soon after pollination may improve fruit and seed set after interspecific pollination. In many crosses, application of growth substances promotes post-pollination development up to a stage when hybrid embryos can be excised and cultured. Immunosuppressors such as amino-n-caproic acid, salicylic acid and acriflavin have also been used to produce wide hybrids in many cereals.

A number of in vitro methods have also been developed to overcome post-fertilization barriers in crop plants. When abortion occurs in a very young stage and maternal tissue has no negative influence on the development of seeds, ovary culture can be applied. When the mismatch between embryo and endosperm development starts very early and ovary culture and/or ovary slice culture fails, ovules can be dissected out of the ovaries and cultured in vitro. In wide crosses where few embryos are produced, the efficiency to recover viable hybrid plants may be enhanced by callus induction from the embryo and subsequent regeneration of plantlets (Pratap et al. 2010). Embryo culture can be applied successfully in crosses in which pollinated flowers can stay on the plant for a notable time, before natural abscission occurs.

1.4 Horizontal Gene Transfer

Horizontal gene transfer (HGT), also referred to as lateral gene transfer (LGT), is a process in which a recipient organism acquires genetic material from a donor organism by asexual means (Bock 2009). Therefore, it is the transfer of genes between the non-mating species and is not restricted by genome or gene pool boundaries. HGT is an adaptive force in avolution, contributing to metabolic, physiological and ecological innovation in most prokaryotes and some eukaryotes (Ragan and Beiko 2009). HGT is primarily associated with prokaryotic species (Johnsborg et al. 2007; Scudellari 2011) and it is expected to contribute up to 10–20 % of the genes in them (Nakamura et al. 2004) modifying important traits such as photosynthesis, nitrogen fixation, virulence and antibiotic resistance. However, this phenomenon has also been reported to occur in plant species on a large scale (Richardson and Palmer 2007; Bock 2009). The most classical example of HGT in plants is the infection of plant cells with *Agrobacterium*. During infection, a region of the Ti plasmid (tumour-inducing plasmid) of the bacterium is incorporated in the nuclear genome of the plant cell (for review see Gelvin 2000). This phenomenon leads to development of modified Ti plasmids which are used as vehicles for introducing foreign genes into plants via *Agrobacterium*-mediated genetic transformation.

Considerable efficiency and skill have now been achieved in transferring exogenous DNA into plants and achieving their expression.

Horizontal transfer of transposable elements in vascular plants was first reported in *Setaria* where a Mu-like element (MULE) has a striking similarity to a MULE in the rice (*O. sativa*) genome (Diao et al. 2006). Since *Setaria* and rice or their relatives do not engage in epiphytotic or parasitic relationship and natural grafting does not occur in monocotyledonous plants, it was suggested that the horizontal transfer of the MULE transposon was mediated by some vector, perhaps a pathogen or an insect pest which was common to both *Setaria* and rice. Another example of HGT has been reported in the grass *Festuca ovina* where a gene encoding the enzyme phosphoglucose isomerase is most closely related to phosphoglucose isomerase genes in the reproductively separated grass genus Poa (Vallenback et al. 2008). Based on synonymous substitutions it has been estimated that the HGT from *P. palustris* or a closely related Poa species to *F. ovina* occurred >600,000 years ago (Vallenback et al. 2008).

HGT has also been reported to occur between plants' mitochondrial genes (Richardson and Palmer 2007). Mitochondria have a unique feature in that they have an active homologous recombination system and they readily undergo fusion (Arimura et al. 2004; Carlsson et al. 2007). This makes them particularly receptive to the horizontal exchange of DNA (Bock 2009). Several studies have documented frequent HGT of mitochondrial DNA sequences between distantly related vascular plant species (Bergthorsson et al. 2003; Won and Renner 2003; Davis and Wurdack 2004; Davis et al. 2005). There is also an evidence of gene transfer from plastid genome of an unidentified plant to the mitochondrial genome of a Phaseolus species (Woloszynska et al. 2005). It was also reported that the genome of a eudicot parasite, *Striga hermonthica*, contains a nuclear gene that is widely conserved among grass species but is not found in other eudicots. Phylogenetically these cluster with Sorghum, the monocot host of the parasite, suggesting that the nuclear genes can be captured by parasitic weeds in the nature (Yoshida et al. 2010).

1.4.1 Transgene Introgression

The infection of plant cells with *Agrobacterium* is a classic example of plants as recipients of HGT between kingdoms (Bock 2009). In case of transgenics the transferred gene usually derives from an alien species that is neither the recipient species nor a close, sexually compatible relative (Schouten et al. 2006a). The ability to transform crop plants has developed tremendously since the first transformed plants were reported in 1983.

There are mainly two approaches of plant transformation: (i) the use of *Agrobacterium* as a biological vector for foreign gene transfer and (ii) direct gene transfer techniques wherein the DNA is introduced into cells by the use of physical, chemical and electrical methods. *Agrobacterium*-based methods are simple and more efficient but have the disadvantage that these are not applicable in all plant

species (Pratap et al. 2009b). However, the host-specific limitations have been largely overcome in many plant species by developing specific cell culture procedures and refining inoculation and co-cultivation. On the other hand, direct gene transfer methods are species and genotype independent in terms of DNA delivery, though their efficiency is affected by the target cell as well as the ease of regeneration from the target cells. Chapter 5 describes the different approaches of alien gene transfer through transgenesis in detail.

Since the introduction of transgenic crops, the area under these crops has increased tremendously. The first-generation transgenics concentrated mainly on tolerance to herbicide and insect-pest larvae in the crops, mainly soybean, maize, cotton and canola. The second-generation transgenics are now focusing on transfer of genes for quality traits as well as drought tolerance and higher nitrogen use efficiency. While the genetically modified crops have been highly successfull throughout the globe, the GM technology has also encountered substantial scepticism among the general public and consequently among the farmers. This is mainly due to the apprehension that transgenes may persist in the environment in wild and weedy unintended hosts and have negative ecological consequences. Studies have shown that one of the major concerns of the public about the transgenic crops is artificial combination of genetic elements derived from different organisms that cannot be crossed by natural means (Lassen et al. 2002; Bauer and Gaskell 2002). Transgene dispersal from the genetically modified crop into the wild relative is as simple as pollen from one plant to another plant or one crop to another crop. However, transgene flow does not necessarily mean introgression and it may require several generations of hybridizations and backcrossings for the introgression of transgene to occur. Furthermore, several other issues are still there which remain to be addressed, particularly, improving the frequency of transformation, increasing the number of genes that can be transferred, better control of expression of the transferred genes and enabling the genes to be inserted at definite positions.

1.5 Somatic Hybridization

Somatic hybridization has been applied for improvement of cultivated plant species as genes can be transferred by protoplast fusion against bacterial, fungal and virus diseases or even nematodes and abiotic stresses such as drought, cold and soil salinity (Göntér et al. 2002). Somatic hybridization provides the breeders the possibility of accessing sexually incompatible germplasm between the crop species and distant relatives, merging genomes of sexually dysfunctional cultivars or breeding lines and substituting one cytoplasm for another with a little effect on nuclear genome (Johnsson and Veilleux 2001). For protoplast fusion technology to be successful two criteria must be fulfilled: protoplast must be isolated in large quantities, and the isolated protoplasts must be totipotent (Waara and Glimelius 1995). Initially successful somatic hybridization was reported through the use of polyethylene glycol (PEG)-mediated fusion (Kao and Michayulk 1974; Wallin et al. 1974).

This was followed by electrofusion techniques (Bates and Hasenkampf 1985; Puite et al. 1985; Fish et al. 1988).

The greatest potential for the use of protoplasm fusion or somatic hybridization lies in creating new crop varieties containing the nuclear genome of one species in the background of the cytoplasmic background of another or in a mixed cytoplasm with organelles from both the species (cybrids). If the complete genomes of two different species are combined parasexually, the resultant hybrid is known as amphidiploid somatic hybrid. Protoplasts from different species can be induced to fuse by exposure to certain chemicals or electric current (Goodman et al. 1987). The resulting somatic hybrid may be grown in vitro to produce callus tissue from which a whole plant can be regenerated, depending upon the species.

Interspecific somatic hybrids are mostly polyploid and often contain many unwanted traits derived from the wild or the unadapted species (Waara and Glimelius 1995). This necessitates several rounds of backcrossing of the somatic hybrid with the cultivated species to remove the undesirable characteristics as well as to establish the optimum ploidy level for crop production. Therefore, unless both the fusion partners are adapted species, the resultant hybrid may not be expected to carry all the desirable alleles.

In an effort to limit the genetic contribution of an unadapted parent to the product of protoplast fusion, some geneticists have promoted asymmetric somatic hybridization (Johnsson and Veilleux 2001). Here the genome of the donor species is fractioned by irradiation prior to fusion which leads to retention of complete genome of the recipient species and only fragments of the genome of donor species. However, since damage to the genome as a result of irradiation is random, transmission of the trait of interest is not always certain. In many instances where two completely distant genomes have been combined, the resulting hybrids have been found to be sterile.

Since the major objective of somatic hybridization is to transcend mating barriers, the majority of somatic hybridization research has been conducted to obtain interspecific somatic hybrids. Consequently a large number of somatic hybrids have been generated in the families Rutaceae, Solanaceae, Brassicaceae, Fabaceae, Asteraceae, Liliaceae and Cucurbitaceae (for review, see Waara and Glimelius 1995; Johnsson and Veilleux 2001). Similarly intergeneric somatic hybrids have also been produced through somatic hybridization. Intergeneric somatic hybrids generally bridge a much wider gap between the two fusion partners than intra- or interspecfic fusion. The most noticeable cases of intergeneric hybrids have been those of *Lycopersicon esculentum* + *S. tuberosum* (Wolters et al. 1994, 1995), *L. esculentum* + *Nicotiana tabacum* (Hossain et al. 1994), *Brassica oleracea* + *Camelina sativa* (Hansen 1998; Sigareva and Earle 1999), *B. oleracea* + *Sinapis alba* (Hansen and Earle 1997), *B. juncea* + *Diplotaxis catholica* (Kirti et al. 1995), *Hordeum vulgare* + *Daucus carota* (Kisaka et al. 1997), *Oryza sativa* + *H. vulgare* (Kisaka et al. 1998) and *O. sativa* + *Lotus corniculatus* (Nakajo et al. 1994).

Apart from intra- and interspecific and intergeneric hybrids, asymmetric somatic hybrids have also been developed by the fusion of protoplasts with unequal genetic contributions from two fusion partners. This has been mostly tried in cases where

one of the fusion partners is a wild or an unadapted species. Although combining distantly related or unadapted species results in asymmetric hybrids which have reduced fertility, these can be employed as bridge species for transfer of alien genes through sexual methods.

Asymmetric somatic hybrids have been sought in attempts to introduce only a part of the genome of a wild (donor) species into the cultivated species (recipient) to limit the amount of alien germplasm to smaller genomic sectors that may control a trait of interest (Johnsson and Veilleux 2001). Since chromosomal rearrangements and eliminations seem to be random, selection for the specific desirable genes would be a more practical approach for better utilization of asymmetric hybrids. The asymmetric hybrids may be induced by irradiation of the donor genome prior to fusion. Most of the examples of utilization of asymmetric hybridization come from Solanaceae, Brassicaceae, Poaceae and Fabaceae. In another method known as cybridization, the plastome and/or chondriome of one crop species is combined with the nuclear genome of another species. This technique has been used to transfer genes for economically important traits such as cytoplasmic male sterility between species (Matibiri and Mantell 1994; Zubko et al. 1996; Sigareva and Earle 1997). The cytoplasm of a species can be replaced in a single step through this technique thereby saving time and resources for transfer of alien cytoplasms through repeated backcrossings.

Somatic hybridization has exhibited great potential in transfer of alien genes, particularly in these species which have sexual incompatibility. In most of the cases, however, traditional breeding was required to intervene between the actual protoplast fusion event and development of a product. Problems with regeneration and differentiation of plants from the protoplasts are still problem in many species. From practical point of view however, gene transfer through somatic hybridization has been much of academic interest.

1.6 Intragenesis and Cisgenesis

With the aim of meeting reservations of general public associated with the development and use of transgenic crop varieties and at the same time ensuring an environmentally sound and efficient plant production, the two transformation concepts, intragenesis and cisgenesis, were developed as alternatives to transgenic crop development (Holme et al. 2013). This grouping of genetically modified plants operates on the basis of phylogenetic distances between the DNA donor source and the recipient species.

The different categories of genetically modified crops were first described by Nielsen (2003) on the basis of such phylogenetic distances. The category with the shortest distances comprised only the plants modified with DNA from the same sexual compatibility group and was termed intragenic (Holme et al. 2013). The cisgenesis concept introduced by Jochemsen and schouten in 2000 (Jochemsen 2000) was initially limited to the derivation of genes to be transferred from the

species itself; subsequently, it was extended to the entire gene pool of the sexually compatible species (Schouten et al. 2006a, b). In cisgenesis, the gene of interest (i.e. cisgene), containing its native introns, promoters and terminator in sense orientation, is taken from the species itself or a sexually compatible relative for genetic transformation. Hence, the gene pool exploited in cisgenesis is identical to the gene pool available for traditional breeding. However, since foreign genes such as the selection marker genes are absent or eliminated from the primary intragenic/cisgenic transformant or their progeny, the crops become far more acceptable by the general public. Cisgenic plants have no extra risks as compared to plants from conventional breeding or mutation breeding (Schouten et al. 2006b). Cisgenesis may be particularly useful in introgression of targeted desirable gene(s) of wild plants into the recipient genome in a single step. Therefore there are no chances of linkage drag (i.e. transfer of other undesirable gene), which is usually a problem in case of traditional breeding. Therefore, it is evident that intragenesis and cisgenesis do not alter the gene pool of the recipient species and practically do not offer any changes in fitness that could not occur through artificial or natural hybridization in the crop species. The advantage these techniques offer is the speed by which a gene can be transferred into the crop plant which can save considerable time of a breeder in developing a superior cultivar.

1.7 Detection of Alien Gene Transfer

In any alien gene transfer process where sexual hybridization is involved, it becomes very important to keep track of the validity of the wide hybrids and also the actual retention of the alien chromatin during generation advancement (Chaudhary et al. 2011). The extent and amount of gene transfer from wild relative into a crop species are determined by the breeding system of the plants (Hancock et al. 1996) as well as fulfillment of the prerequisites for hybrid formation and its survival in the environment. Gene flow between species resulting in permanent exchange of genes from one set of differentiated population to another, i.e. incorporation of alleles into a new, reproductively integrated population system, is known as introgression. Nevertheless, the events of convergence, independent mutation and sharing of genes from a common ancestor cannot be rejected in estimating the role and consequences of gene introgression (Rieseberg and Brunsfeld 1992). For successful detection of an alien gene a prior knowledge of the phenotypic effects of a gene is essential. Further, the gene sequence or the protein being targeted must express in the hybrid population. Hybridization and introgression are often difficult to detect and are not necessarily indicated by the occurrence of characters of one taxon in another (Donald and Hamblin 1983). Detection of gene transfer is still more difficult in high-frequency outcrossing crops where a few detectable traits separate the crop from its wild relative. Sometimes the efforts of detecting successful hybridization on the basis of morphological characters of hybrids may be misleading as the crops and wild relatives may possess similar characters due to common ancestor or as a

result of symplesiomorphy or convergent evolution (Nason et al. 1992; Wilson 1992). Most of the introgressions generally appear to be localized and dispersed. Therefore, it is also likely that many cases of low-level introgression are not detected (Rieseberg and Brunsfeld 1992). However, if an allele that is characteristic of one species is found in another species, with its highest frequencies near the hybrid zone, introgression is likely to have occurred (Harrison 1990).

The use of molecular markers has tremendously increased our ability to detect and quantify alien gene transfer. DNA markers have undoubtedly provided the most robust methods of alien gene detection in crop plants. In the advanced backcross QTL (AB-QTL) approach, parallel discovery and transfer of desired QTL from an unadapted germplasm into selected breeding lines take place (Tanksley and Nelson 1996). In this approach, repeated backcrossing is done with the elite parent in wild × cultivated species cross, and selection is imposed in advanced backcrossed (BC_2F_2 or BC_2F_3) populations (Pratap et al. 2013). This approach besides reducing linkage drag also generates phenotyping and genotyping data. The advanced backcross populations are simultaneously used to identify desirable genes/QTL through QTL analysis. Once favourable QTL alleles are identified, a few marker-assisted selection generations (3–4) can lead to development of near-isogenic lines (NILs) which can be used for development of a variety. This approach has been successfully used in soybean and commonbean (Blair et al. 2003; Chaky et al. 2003). In pigeonpea, a set of 17 amphiploid and autotetraploid groundnuts has been developed (Mallikarjuna et al. 2011).

In another approach, introgression libraries are constructed made up of several introgression lines (ILs) which are developed by repeated backcrossing of F_1s between wild and cultivated lines (see Pratap et al. 2013). This leads to the distribution of donor (wild species) genome into the entire genome of ILs and consequently their expression in the phenotype. Such libraries have been reported in soybean using wild soybean species (*G. soja*) (Concibido et al. 2003) and peanut from synthetic tetraploids (Foncéka et al. 2009).

In situ hybridization is now recognized as another important technique to locate the physical position of a known DNA sequence on a chromosome. Methods of in-situ hybridization have made it feasible to link the molecular data about DNA sequence with chromosomal and expression information at the tissue, cellular and sub-cellular level and hence changed the way we apply cytogenetics to agriculture (Schwarzacher and Heslop-Harrison 2000). Since the first application to identify chromosomes (Schwarzacher et al. 1989) and visualize DNA sequences on plant chromosomes (Yamamoto and Mukai 1989), genomic in situ hybridization (GISH) and fluorescence in situ hybridization (FISH) are now the preferred techniques to physically visualize genome and chromosomes and the order of chromosome segments, genes and DNA sequences. In the past two decades, many applications and refinements have been made in the technology which opened new vistas for microscopic visualization of DNA manifestation in situ, previously confined to gel blot hybridization. Simultaneous detection of multiple targets has become quite easy through multicolour FISH and has been exercised in various cereal plants, viz., rye (Leitch et al. 1991), wheat (Mukai et al. 1993; Komeda et al. 2007; Chaudhary

2008, 2009; Chaudhary et al. 2009), barley (Leitch and Heslop-Harrison 1993), *Aegilops* (Yamamoto and Mukai 1995), triticale (Cuadrado and Jouve 1994), chickpea (Staginnus et al. 2001), lentil (Galasso 2003), *Phaseolus* (Moscone et al. 1999) and *Vigna* spp. (Khattak et al. 2007). Therefore, the innovative techniques of molecular cytogenetics can be reliably utilized in various crop plants to physically map the whole genomes and the targeted alien introgressions to resolve various issues related to the origin of the species, assessment of variability and physical mapping at chromosomal level.

At certain instances combination of more than one approach may be discernible to detect alien gene transfer. Direct observation of characteristics that appear to derive from another taxa can be combined with other analytical studies that provide evidence of introgression (Jarvis and Hodgkin 1999). For example, molecular, cytogenetic and agro-morphological studies can be combined with field studies by agronomists, ecologists, anthropologists and other social scientists to provide a data supporting the likelihood of alien gene introgression (Jarvis and Hodgkin 1999).

1.8 Gene Transfer from Domesticated Plants to Their Wild Relatives

Over the last two decades, studies on the effects of crop-to-wild plant hybridization and transfer of genes have been receiving much attention. Most domesticated plants hybridize naturally with their cross-compatible wild relatives, when they come into contact (Ellstrand et al. 1999). Several instances of introgression between wild and domesticated populations have demonstrated that gene transfer occurs in both the directions: from domesticated to wild and wild to domesticated (Ellstrand et al. 1999; Jarvis and Hodgkin 1999). While the gene flow from wild to domesticated crop plants can be achieved through several modern crop breeding and genetic tools such as distant hybridization aided by embryo rescue and in vivo hormone applications, in farmers' fields it can occur only when farmers use part of their crop produce as seed for the next generation of sowing without replacing the seed with that of the commercial varieties. However since this is becoming a rare phenomenon in modern agriculture the gene flow mostly occurs from domesticated to wild plants. Papa and Gepts (2003) demonstrated from admixture population in commonbean that gene flow between wild and domesticated population leads to asymmetric introgression, with a higher rate from domesticated to wild populations. In several other studies also, it was demonstrated that gene flow was higher from domesticated to wild populations (Wolfe et al. 2001; Matsuoka et al. 2002).

Gene flow can occur from crop to crop, crop to a wild relative and even vice versa. However, in all cases it is not the gene flow per se, rather the type of genes and their utilization in creation of variability, thereby providing avenues for selection, introgression of new traits and its effects on the recipient population which have been important for the breeders and the mankind. Landraces are an important source of genetic diversity. Many of the landraces are still being cultivated within their

centres of origin. Local farmers play an important role in the maintenance of in situ diversity and conservation (Gepts and Papa 2003). Since the inception of systematic plant breeding efforts, there has been use of limited genetic resources that has led to inadvertent narrowing down of genetic base of most of the crop plants.

There are several instances where transgene flow cases have been reported in centres of origin and diversity of crops. One of the most notable examples is of transgene introgression of maize in Mexico (Quist and Chapela 2001, 2002). Even while the paper was retracted by Nature since introgression per se was not shown, this has led to concerns for similar transgene introgressions in other important crop plants such as rice and soybean in China (Huang et al. 2003). However, transgene dispersal from GM crops to wild relatives is often simply seen as pollen flow from the crop to wild relative. Nevertheless, the process of introgression is not so simple and actually occurs in may steps that involve several hybrid generations, all of which can exchange genes and coexist simultaneously for many years (Neal Stewart et al. 2003).

The processes and outcomes of hybridization have been well explained, and historically the fundamentals can be traced back to Linnaeus who proposed a model of speciation by hybridization (Arnold 1997). It is now well acknowledged that hybridization has played an important role in the evolution of crop plants (Abbott 1992; Riesberg and Ellstrnd 1993; Ellstrand et al. 1996).

1.9 Conclusions and Future Prospects

Gene transfer in crop plants, mostly through traditional plant breeding and more recently aided by transgenesis and molecular marker technology, has proved to be one of the most powerful tools for crop improvement and has increased the yield levels of crop plants to the present level which is well supporting the ever-increasing population of the world. The plants have evolved in the nature along with the evolution of human civilization. While natural evolution has been governed by the environmental forces, the human interference has hastened the process of evolution of crop species, particularly those which are of use to him either directly or indirectly. The transition of human race from a collector of seeds to a producer of grains led to more and more interaction between him and the plants which have been useful to him. This transition has travelled a very long way from unknowing attempts of domestication to crop breeding to genetic transformation culminating into present-day super domestication. Genetic diversity of crop plants, either natural or man-made, played significant role in this evolution. Useful traits such as resistance to diseases and insect pests, tolerance to abiotic stresses, improvement of quality traits and improved plant types have been transferred to crop varieties from cultivated and non-cultivated backgrounds. For this purpose, intraspecific, interspecific and even intergeneric gene transfers are not new, while technologies like somatic hybridization and protoplast fusion have also been helpful in combining desirable genes from two different sexually incompatible partners. The advent of recombinant DNA technology however drew greater attention of the scientific community

as well as general public which has recently been supplemented by intragenesis and cisgenesis. The broad potential of recombinant DNA technology has provided the possibility of both molecular analysis of crop productivity as well as ways in which it may be possible to increase the productivity.

While at present we may be complacent about food production, thanks to the genomic revolution, the predicted growth in the world population, eroding genetic diversity, decreasing water availability and the effects associated with predicted climate change pose challenges to modern agriculture. Nevertheless, these challenges encourage us to utilize the vast amount of knowledge generated and the available gene transfer technologies towards development of "super plant types" which fit well in our imagination of a perfect plant species. Improved genetic maps as well as molecular cytogenetic tools may allow for gene isolation from microdissected chromosomes and their easy detection in the recipient species. These technologies need to be vigorously taken up for isolation and transfer of poorly characterized genes such as those governing resistance to abiotic stresses, disease resistance, quality traits, etc. Advances in mapping technologies and molecular markers can speed up the discovery and characterization of genes for complex traits. Sequencing of mitochondrial genomes of those plants where HGT is known to have occurred will uncover transfers that are too short or are from such evolutionary distant donors that they fail to amplify by PCR (Richardson and Palmer 2007). It also needs to be established whether multigenes can be transferred simultaneously or at least from the same donor lineage through HGT. Transgenic approach while having great utility in overcoming genome barriers for gene transfer is also associated with general public scepticism. Techniques like cisgenesis and transgenesis both have wider acceptability and a great potential to overcome some of the limitations of classical breeding including linkage drag as well as genetic transformation. Such techniques may have an increased utility in less studied and highly environment-sensitive crops such as food legumes where gene transfer through repeated backcrossing is difficult. Crop breeding has now reached a stage where development of super-domesticates with traits such as dramatically increased crop yield, resistance to multiple diseases and insect-pests, bio-fortified grains and even radically changed crops such as conversion of C3 crops into C4 crops are not unrealistic. A greater interdisciplinary contribution from breeders, biotechnologists, plant physiologists, pathologists, nutritionists and ecologists together will be able to achieve the true potential of alien gene transfer in realizing a food revolution to make this world a hunger-free planet.

References

Abbott RJ (1992) Plant invasions, interspecific hybridization and the evolution of new plant taxa. Trends Ecol Evol 7:401–405

Altieri MA, Montecinos C (1993) Conserving crop genetic resources in Latin America through farmers' participation. In: Christopher S, Potter DJ, Cohen JI (eds) Perspectives on biodiversity: case studies of genetic resource conservation and development. American Association for the Advancement of Science (AAAS), Washington, DC, pp 45–64

Anderson E (1949) Introgressive hybridization. Wiley, New York, NY

Anderson E (1961) The analysis of variation in cultivated plants with special reference to introgression. Euphytica 10:79–86

Arimura S, Yamamoto J, Aida GP, Nakazono M, Tsutsumi N (2004) Frequent fusion and fission of plant mitochondria with unequal nucleoid distribution. Proc Natl Acad Sci U S A 101: 7805–7808

Arnold ML (1992) Natural hybridization as an evolutionary process. Annu Rev Ecol Syst 23:237–261

Arnold ML (1997) Natural hybridization and evolution. Oxford University Press, New York

Bates GW, Hasenkampf CA (1985) Culture of plant somatic hybrids following electrical fusion. Theor Appl Genet 70:227–233

Bauer M, Gaskell G (2002) Researching the public sphere of biotechnology. In: Gaskell G, Bauer M (eds) Biotechnology: the making of a global controversy. Cambridge University Press, Cambridge, pp 1–19

Bergthorsson U, Adams KL, Thomason B, Palmer JD (2003) Widespread horizontal gene transfer of mitochondrial genes in flowering plants. Nature 424:197–201

Blair MW, Pedraza F, Buendia HF, Gatian-Soils E, Beebe SE, Gepts P, Tohme J (2003) Development of a genome-wide anchors microsatellite map for common bean (*Phaseolus vulgaris* L.). Theor Appl Genet 107:1362–1374

Blakeslee AF (1945) Removing some of the barriers to crossability in plants. Proc Am Philos Soc 89:561–574

Bock R (2009) The give and take of DNA: horizontal gene transfers in plants. Trends Plant Sci 15:11–22

Brown CR, Adiwilaga KD (1991) Use of rescue pollination to make a complex interspecific cross in potato. Am Potato J 68:813–820

Carlsson J, Lagercrantz U, Sundstrorm J, Teixeira R, Wellmer F, Meyerowitz EM, Glimelius K (2007) Microarray analysis reveals altered expression of a large number of nuclear genes in developing cytoplasmic male sterile *Brassica napus* flowers. Plant J 49:452–462

Chaky JM, Specht JE, Cregan PB (2003) Advanced backcross QTL analysis. In: Proceedings of the plant and animal genome XII conference abstracts, San Diego, CA, USA, 10–14 January 2004, pp. 545

Chaudhary HK (2008) Dynamics of doubled haploidy breeding and molecular cytogenetic approaches in bread wheat: focus on north-west Himalayan regions. Adv Chrom Sci 3:67–69

Chaudhary HK (2009) New frontiers in chromosome engineering: genetic upgradation of bread wheat for varied agroclimatic situations in north-west Himalayas. In: Proceeding of national seminar on designing crops for the changing climate, Ranchi, Jharkhand, India. 30–31 October, 2009, pp. 51–52

Chaudhary HK, Chahota RK, Mukai Y, Jeberson MS, Kishore N, Kumar V (2009) Molecular cytogenetic mapping of the targeted rye chromatin introgressed bread wheat lines associated with drought tolerance and rust resistance suitable for rainfed regions of north-west Himalayas. In: Proceedings of national seminar on designing crops for the changing climate, Ranchi, Jharkhand, India, 30–31 October, 2009, pp. 84

Chaudhary HK, Sood VK, Tayeng T, Kaila V, Sood A (2011) Molecular cytogenetics in physical mapping of genomes and alien introgressions. In: Pratap A, Kumar J (eds) Biology and breeding of food legumes. CABI, Oxfordshire, pp 131–146

Concibido VC, Vallee BL, Mclaird P, Pineda N, Meyer J, Hummel L, Yang J, Wu K, Delannay X (2003) Introgression of a quantitative trait locus for yield from glycine soja into commercial soybean cultivars. Theor Appl Genet 106:575–582

Coyne JA, Orr HA (2004) Speciation. Sinauer Associates, Sunderland, MA

Cuadrado A, Jouve N (1994) Mapping and organization of highly-repeated DNA sequences by means of simultaneous and sequential FISH and C-banding in 6x Triticale. Chromosome Res 2:331–338

Darwin C (1859) On the origin of species by means of natural selection, or the preservation of favored races in the struggle for life. Murray, London

Davis CC, Wurdack KJ (2004) Host-to-parasite gene transfer in flowering plants: phylogenetic evidence from malpighiales. Science 305:676–678
Davis CC, Anderson WR, Wurdack KJ (2005) Gene transfer from a parasitic flowering plant to a fern. Proc Roy Soc B Biol Sci 272:2237–2242
Diao X et al (2006) Horizontal transfer of a plant transposon. PLoS Biol 4:e5
Donald CM, Hamblin J (1983) The convergent evolution of annual seed crops in agriculture. Adv Agron 36:97–143
Ellstrand NC, Whitkus RW, Rieseberg LH (1996) Distribution of spontaneous plant hybrids. Proc Natl Acad Sci U S A 93:5090–5093
Ellstrand NC, Prentice HC, Hancock JF (1999) Gene flow and introgression from domesticated plants into their wild relatives. Annu Rev Ecol Syst 30:539–563
Fish N, Karp A, Jones MGK (1988) Production of somatic hybrids by electrofusion in *Solanum*. Theor Appl Genet 76:260–266
Foncéka D, Hodo-Abalo T, Rivallan R, Faye I, Sall MN, Ndoye O (2009) Genetic mapping of wild introgressions into cultivated peanut: a way toward enlarging the genetic basis of a recent allotetraploid. BMC Plant Biol 9:103
Futuyma DJ (1998) Evolutionary biology. Sinauer Associates, Sunderland, MA
Galasso I (2003) Distribution of highly repeated DNA sequences in species of the genus *Lens* Miller. Genome 46:1118–1124
Gelvin SB (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annu Rev Plant Physiol Plant Mol Biol 51:223–256
Gepts P, Papa R (2003) Possible effects of (trans) gene flow from crops on the genetic diversity from landraces and wild relatives. Environ Biosafe Res 2:89–103
Gill BS, Friebe BR, White FF (2011) Alien introgressions represent a rich source of genes for crop improvement. Proc Natl Acad Sci U S A 108:7657–7658
Göntér I, Szarka B, Lendvai Á, Molnár-Láng M, Mórocz S, Dudits D (2002) Problems and possibilities of wheat-maize somatic hybridization. Proc Hung Cong Plant Physiol 46:11–12
Goodman RM, Hauptli H, Crossway A, Knauf VC (1987) Gene transfer in crop improvement. Science 236:48–54
Hajjar R, Hodgkin T (2007) The use of wild relatives in crop improvement: a survey of developments over the last 20 years. Euphytica 156:1–13
Hancock JF, Grumet R, Hokanson SC (1996) The opportunity for escape of engineered genes from transgenic crops. Horticult Sci 31:1080–1085
Hanelt P (1997) Gene flow between crops and related taxa-some case studies. Bocconea 7:51–61
Hansen LN (1998) Intertribal somatic hybridization between rapid cycling *Brassica oleracea* L. and *Camelina sativa* (L.) Crantz. Euphytica 104:173–179
Hansen LN, Earle ED (1997) Somatic hybrids between *Brassica oleracea* L. and *Sinapis alba* L. with resistance *to Alternaria brassicae* (Berk.) Sacc. Theor Appl Genet 94:1078–1085
Harlan JR, de Wet JMJ (1971) Toward a rational classification of cultivated plants. Taxon 20:509–517
Harrison RG (1990) Hybrid zones: windows on the evolutionary process. In: Futuyama DJ, Antonovics J (eds) Oxford surveys in evolutionary biology, vol 7. Oxford University Press, Oxford, pp 69–128
Hauser TP, Jorgensen RB et al (1998) Fitness of backcross and F_2 hybrids between weedy *Brassica rapa* and oilseed rape (*B. napus*). Heredity 81:436–443
Hoisington D, Khairallah M, Reeves T, Ribaut JM, Skovmand B, Taba S, Warburton M (1999) Plant genetic resources: what can they contribute toward increased crop productivity? Proc Natl Acad Sci U S A 96:5937–5943
Holme IB, Wendt T, Holm PB (2013) Intragenesis and cisgenesis as alternatives to transgenic crop development. Plant Biotech J 11:395–405. doi:10.1111/pbi.12055
Hossain M, Imanishi S, Matsumoto A (1994) Production of somatic hybrids between tomato (*Lycopersicon esculentum*) and night shade (*Solanum lycopersicoides*) by electrofusion. Breed Sci 44:405–412
Huang J, Rozell S et al (2003) Plant biotechnology in China. Science 295:674–676

Iltis HH (1988) Serendipity in the exploration of diversity. In: Wilson EO (ed) Biodiversity. Natl. Acad. Press, Washington, DC, pp 98–105

Jarvis DI, Hodgkin T (1998) Wild relatives and crop cultivars: conserving the connection. In: The proceedings of an international symposium on in situ conservation of plant genetic diversity, Zencirci N, Kaya Z, Anikster Y, Adams WT (eds), pp. 73–80, Central Research Institute for Field Crops, Ankara, Turkey

Jarvis DI, Hodgkin T (1999) Wild relatives and crop cultivars: detecting natural introgression and fermer selection of new genetic combinations in agroecosystems. Mol Ecol 8:S159–S173

Jauhar PP (1993) Alien gene transfer and genetic enrichment of bread wheat. In: Damania AB (ed) Biodiversity and wheat improvement. ICARDA-AWiley Sayce Publication, Aleppo, pp 103–119

Jauhar PP, Chibbar RN (1999) Chromosome mediated and direct gene transfers in wheat. Genome 42:570–583

Jiang J, Friebe B, Gill BS (1994) Recent advances in alien gene transfer in wheat. Euphytica 73:199–212

Jochemsen H (2000) Toetsen en begrenzen. Een ethische en politieke beoordeling van de moderne biotechnologie, Wetenschappelijke Studiecentra van RPF en GPV (ChristenUnie)

Johnsborg O, Eldholm V, Harvarstein LS (2007) Natural genetic transformation: prevalence, mechanisms and function. Res Microbiol 158:767–778

Johnsson AAT, Veilleux RE (2001) Somatic hybridization and applications in plant breeding. Plant Breed Rev 20:167–226

Kao KN, Michayulk MR (1974) A method of high-frequency intergeneric fusion of plant protoplasts. Planta 115:355–367

Khattak GSS, Wolny E, Saeed I (2007) Detection of ribosomal DNA sites in Chickpea (*Cicer arietinum* L.) and Mungbean (*Vigna radiata* (L). Wiltzek) by fluorescence *in situ* hybridization. Pak J Bot 39:1511–1515

Kirti PB, Mohapatra T, Khanna H, Prakash S, Chopra VL (1995) *Diplotaxis catholica*+*Brassica juncea* somatic hybrids: molecular and cytogenetic characterization. Plant Cell Rep 14:593–597

Kisaka H, Kisaka M, Kanno A, Kameya T (1997) Production and analysis of plants that are somatic hybrids of barley (*Hordeum vulgare* L.) and carrot (*Daucus carota* L.). Theor Appl Genet 94:221–226

Kisaka H, Kisaka M, Kanno A, Kameya T (1998) Intergeneric somatic hybridization of rice (*Oryza sativa* L.) and barley (*Hordeum vulgare* L.) by protoplast fusion. Plant Cell Rep 17:362–367

Knott DR, Dvorak J (1976) Alien germplasm as a source of resistance to diseases. Annu Rev Plant Physiol Plant Mol Biol 14:211–235

Komeda N, Chaudhary HK, Suzuki G, Mukai Y (2007) Cytological evidence for chromosome elimination in wheat×*Imperata cylindrica* hybrids through fluorescence *in situ* hybridization. Gen Genet Syst 82:241–248

Kumar J, Choudhary AK, Solanki RK, Pratap A (2011a) Towards marker-assisted selection in pulses - a review. Plant Breed 130:297–313

Kumar S, Mohammad I, Gupta S, Pratap A (2011b) Distant hybridization and alien gene introgression. In: Pratap A, Kumar J (eds) Biology and breeding of food legumes. CAB International, Oxfordshire, pp 81–110

Ladizinsky G, Pickersgill B, Yamamoto K (1988) Exploitation of wild relatives of the food legumes. In: Summerfield RJ (ed) World crops, cool season food legumes. Kluwer Academic Publishers, Dordrecht, pp 967–987

Lassen J, Madsen KH, Sandøe P (2002) Ethics and genetic engineering – lessons to be learned from GM foods. Bioprocess Biosyst Eng 24:263–271

Leitch IJ, Heslop-Harrison JS (1993) Physical mapping of four sites of 5S rDNA sequences and one site of the *a-amylase-2* gene in barley (*Hordeum vulgare*). Genome 36:517–523

Leitch IJ, Leitch AR, Heslop-Harrison JS (1991) Physical mapping of plant DNA sequences by simultaneous *in situ* hybridization of two differently fluorescent probes. Genome 34:329–333

Mallet J (2005) Hybridization as an invasion of the genome. Trend Ecol Evol 20:229–237

Mallikarjuna N, Senapathy S, Jadhav DR, Saxena KB, Sharma HC, Upadhyaya HD (2011) Progress in the utilization of *Cajanus platycarpus* (Benth.) Maesen in pigeonpea improvement. Plant Breed 130:507–514

Matibiri EA, Mantell SH (1994) Cybridization in *Nicotiana tabacum* L. using double inactivation of parental protoplasts and post-fusion selection based on nuclear –encoded and chloroplast –encoded marker genes. Theor Appl Genet 88:1017–1022

Matsuoka Y, Vigouroux Y, Goodman MM, Jesus Sanchez G, Buckler E, Doebley J (2002) A single domestication for maize sown by multilocus microsatellite genotyping. Proc Natl Acad Sci U S A 30:6080–6084

Mayr E (1963) Animal species and evolution. Harvard University Press, London

Moscone EA, Klein F, Lambrou M, Fuchs J, Schweizer D (1999) Quantitative karyotyping and dual-color FISH mapping of 5S and 18S–25S rDNA probes in the cultivated *Phaseolus* species (Leguminosae). Genome 42:1224–1233

Mukai Y, Nakahara Y, Yamamoto M (1993) Simultaneous discrimination of the three genomes in hexaploid wheat by multicolour fluorescence *in situ* hybridization using total genomic and highly repeated DNA probes. Genome 36:489–494

Nakajo S, Niizeki M, Harada T, Ishikawa R, Saito K (1994) Somatic cell hybridization in rice (*Oryza sativa* L.) and birdsfoot trefoil (*Lotus corniculatus* L.). Breed Sci 44:79–81

Nakamura Y, Itoh T, Matsuda H, Gojobori T (2004) Biased biological functions of horizontally transferred genes in prokaryotic genomes. Nat Genet 36:760–766

Nason JD, Ellstrand NC, Arnold ML (1992) Patterns of hybridization and introgression in populations of oaks, manzanitas and irises. Am J Bot 79:101–111

Neal Stewart C Jr, Halfhill MD, Warwick SI (2003) Transgenic introgression from genetically modified crops to their wild relatives. Nat Rev Genet 4:806–817

Nielsen KM (2003) Transgenic organisms - time for conceptual diversification? Nat Biotechnol 21:227–228

Papa R, Gepts P (2003) Asymmetry of gene flow and differential geographical structure of molecular diversity in wild and domesticated common bean (*Phaseolus vulgaris* L.) Mesoamerica. Theor Appl Genet 106:239–250

Pratap A, Gupta SK (2009a) Biology and Ecology of wild crucifers. In: Gupta SK (ed) Biology and breeding of crucifers. CRC Press, Boca Raton, FL, pp 37–68

Pratap A, Gupta SK (2009b) Biotechnological interventions in host plant resistance. In: Peshin R, Dhawan AK (eds) Integrated pest management: innovation, dissemination and impact. Springer, Dordrecht, pp 183–207

Pratap A, Choudhary AK, Kumar J (2010) *In vitro* techniques towards genetic enhancement of food legumes - a review. J Food Legumes 23:169–185

Pratap A, Tomar R, Rajan N, Kumar J, Mathur PB, Malviya N, Anjum TK (2013) Towards enriching genomic resources in legumes. In: Nadarajan N, Gupta S, Gupta DS (eds) Legumes in omics era. Springer, New York, NY

Prescott-Allen C, Prescott-Allen R (1986) The first resource: wild species in the North American economy. Yale University, New Haven, CT

Prescott-Allen C, Prescott-Allen R (1988) Genes from the wild: using wild genetic resources for food and raw materials. International Institute for Environment and Development, London

Puite KJ, Van Wikselaar P, Verhoeven H (1985) Electrofusion, a simple and reproducible technique in somatic hybridization of *Nicotiana plumbaginifolia* mutants. Plant Cell Rep 4:274–276

Quist D, Chapela IH (2001) Transgenic DNA introgressed into traditional maize landraces in Oaxaca, Mexico. Nature 414:541–543

Quist D, Chapela IH (2002) Maize transgene results in Mexico are artifacts-reply. Nature 416:602

Ragan MA, Beiko RG (2009) Lateral genetic transfer: open issues. Philos Trans R Soc 364: 2241–2251

Repellin A, Baga M, Jauhar PP, Chibbar RN (2001) Genetic enrichment of cereal crops via alien gene transfer: new challenges. Plant Cell Tissue Org Cult 64:159–183

Richardson AO, Palmer JD (2007) Horizontal gene transfer in plants. J Exp Bot 58L:1–9

Rick CM (1974) High soluble solids content in large fruited tomato lines derived from a wild green fruited species. Hilgardia 42:492–510

Riesberg LH, Ellstrnd NC (1993) What can molecular and morphological markers tell us about plant hybridization? Crit Rev Plant Sci 12:213–241

Rieseberg LH, Brunsfeld SJ (1992) Molecular evidence and plant introgression. In: Soltis DE, Soltis PS, Doyle JJ (eds) Molecular systematics of plants. Chapman and Hall, New York, NY, pp 151–176

Riley R, Chapman V, Johnson R (1968) Introduction of yellow rust resistance of *Aegilops comosa* into wheat by genetically induced homeologus recombination. Nature 216:383–384

Sarr A, Sandmeier M, Pernes J (1988) Gametophytic competition in pearl millet, *Pennisetum typhoides* (Stapf et Hubb). Genome 30:924–929

Schouten HJ, Krens FA, Jacobsen E (2006a) Do cisgenic plants warrant less stringent oversight? Nat Biotechnol 24:753

Schouten HJ, Krens FA, Jacobsen E (2006b) Cisgenic plants are similar to traditionally bred plants. EMBO Rep 7:750–753

Schwarzacher T, Heslop-Harrison JS (2000) Practical *in-situ* hybridization. Bios, Oxford, pp 203–XII

Schwarzacher T, Leitch AR, Bennett MD, Heslop-Harrison JS (1989) *In situ* localization of parental genomes in a wide hybrid. Ann Bot 64:315–324

Scudellari M (2011) Gene swap key to evolution. The scientist, http://classic.the-scientist.com/news/display/57962/

Sears ER (1956) Transfer of leaf-rust resistance from *Aegilops umbelluta* to wheat. Brookhaven Symp Biol 9:1–21

Sigareva MA, Earle ED (1997) Direct transfer of a cold tolerant Ogura male –sterile cytoplasm into cabaggae (*Brassica olaracea* ssp. *capitata*) via protoplast fusion. Theor Appl Genet 94:213–220

Sigareva MA, Earle ED (1999) Camelexin induction in intertribal somatic hybrids between *Camelina sativa* and rapid-cycling *Brassica oleracea*. Theor Appl Genet 98:164–170

Slatkin M (1987) Gene flow and the graphic structure of natural populations. Science 236: 787–792

Staginnus C, Huettel B, Desel C, Schmidt T, Kahl G (2001) A PCR-based assay to detect En/Spm-like transposon sequences in plants. Chromosome Res 9:591–605

Stalker HT (1980) Utilization of wild species for crop improvement. Adv Agron 33:111–147

Stebbins GL (1958) The inviability, weakness and sterility of hybrids. Adv Genet 9:147–215

Stebbins GL (1959) The role of hybridization in evolution. Proc Am Philos Soc 103:231–251

Tanksley SD, McCouch SR (1997) Seed banks and molecular maps, unlocking genetic potential from the wild. Science 277:1063–1066

Tanksley SD, Nelson JC (1996) Advanced backcross QTL analysis: a method for the simultaneous discovery and transfer of valuable QTLs from unadapted germplasm into elite breeding lines. Theor Appl Genet 92:191–203

Vallenback P et al (2008) Origin and timing of the horizontal transfer of a *PgiC* gene from Poa to *Festuca ovina*. Mol Phylogenet Evol 46:890–896

Waara S, Glimelius K (1995) The potential of somatic hybridization in crop breeding. Euphytica 85:217–233

Wallin A, Glimelius K, Eriksson T (1974) The induction of aggregation and fusion of *Daucus carota* protoplasts by polyethylene glycol. Z Pflanzenphysiol 74:64–80

Wilson P (1992) On inferring hybridity from morphological intermediacy. Taxon 41:11–23

Wolfe DE, Takebayashi N, Risenberg LH (2001) Predicting the risk of extinction through hybridization. Conserv Biol 15:1039–1053

Woloszynska M, Bocer T, Mackiewicz P, Janska H (2005) A fragment of chloroplast DNA was transferred horizontally, probably from non-eudicots, to mitochondrial genome of *Phaseolus*. Plant Mol Biol 56:811–820

Wolters AMA, Schoenmakers HCH, Kamstra S, Van Eden J, Koornneef M, de Jong JH (1994) Mitotic and meiotic irregularietis in somatic hybrids of *Lycopesicon esculentum* and *Solenum tubersosum*. Genome 37:726–735

Wolters AMA, Schoenmakers HCH, Koornneef M (1995) Chloroplast and mitochondrial DNA composition of triploid and tetraploid somatic hybrids between *Lycopesicon esculentum* and *Solenum tubersosum*. Theor. Appl Genet 90:285–293

Won H, Renner SS (2003) Horizontal gene transfer from flowering plants to Gnetum. Proc Natl Acad Sci U S A 100:10824–10829

Yamamoto M, Mukai Y (1989) Application of fluoroscence *in-situ* hybridization to molecular cytogenetics of wheat. Wheat Inform Service 69:30–32

Yamamoto M, Mukai Y (1995) Physical mapping of ribosomal RNA genes in Aegilops and Triticum. In: Li ZS, Xin ZY (eds), Proceedings of 8th international wheat genetics symposium, pp. 807–811, China: Beijing

Yoshida S, Maruyama S, Nozaki H, Shirasu K (2010) Horizontal gene transfer by the parasitic plant *Striga hermonthica*. Science 328:1128

Zubko MK, Zubko EI, Patskovsky YV, Khvedynich OA, Fisahn J, Gleba YY, Schieder O (1996) Novel ‘homeotic’ CMS patterns generated in *Nicotiana* via cybridization with *Hyoscyamus* and *Scopolia*. J Exp Bot 47:1101–1110

Chapter 2
Distant Hybridization: A Tool for Interspecific Manipulation of Chromosomes

Dengcai Liu, Huaigang Zhang, Lianquan Zhang, Zhongwei Yuan, Ming Hao, and Youliang Zheng

Abstract Wide or distant hybridization has been widely used as an important tool of chromosome manipulation for crop improvement. The chromosome behaviors in F_1 hybrids provide us with the essential genetic basis for chromosome manipulation. The induction of homoeologous pairing in F_1 hybrid plants followed by the incorporation of a single-chromosome fragment from an alien or a wild species into an existing crop species by translocating chromosomes has been used in the production of translocation lines. Most efforts to transfer a beneficial trait from wild plants into crops so far have bridged the species gap via alien chromosome translocation lines. Chromosome doubling in somatic cells or gametes of F_1 hybrids followed by the incorporation of all alien chromosomes has been used in the production of amphidiploids. Amphidiploidy can be used for a bridge to move a single chromosome from one species to another or for the development of new crops. Chromosome elimination of a uniparental genome during the development of F_1 hybrid embryos has been used in the production of haploids. Haploids are very useful in double-haploid breeding of a true-breeding crop such as wheat and rice since this method can quickly replace genetic recombination while enhancing breeding efficiency or facilitating genetic analysis.

Keywords Amphidiploidy • Chromosome manipulation • Gene introgression • Haploid • Translocation

D. Liu, Ph.D. (✉)
Key Laboratory of Adaptation and Evolution of Plateau Biota, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810001, P.R. China

Triticeae Research Institute, Sichuan Agricultural University, Wenjiang, Sichuan, P.R. China
e-mail: dcliu7@yahoo.com

H. Zhang
Key Laboratory of Adaptation and Evolution of Plateau Biota, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810001, P.R. China

L. Zhang • Z. Yuan • M. Hao • Y. Zheng
Triticeae Research Institute, Sichuan Agricultural University, Wenjiang, Sichuan, P.R. China

A. Pratap and J. Kumar (eds.), *Alien Gene Transfer in Crop Plants, Volume 1: Innovations, Methods and Risk Assessment*, DOI 10.1007/978-1-4614-8585-8_2,
© Springer Science+Business Media New York 2014

2.1 Introduction

Wide or distant hybridization, a mating between individuals of different species or genera, provides a way to combine diverged genomes into one nucleus. Wide hybridization breaks what is known as the species barrier for gene transfer and thus makes it possible to transfer the genome of one species to another, which results in changes in genotypes and phenotypes of the progenies. It is very important for species evolution and speciation since chromosome doubling of wide hybrids is responsible for the origin of many allopolyploid species. Repeated backcrossing of wide hybrids to their parental species has also contributed to the evolution and speciation of some species by gene introgression, i.e., the infiltration of chromosomes or chromosome fragments from one species into another through repeated backcrossing of wide hybrids to their parental species (Anderson 1953; Stebbins 1971; Arnold 1997; Mallet 2007). Besides its role in evolution and speciation of species, gene introgression from crops, especially genetically modified crops, into the wild species, may increase the capability of the wild species to adapt to agricultural environments and compete with the cultivated forms, which is viewed as a possible threat to the environment and to agriculture (Ellstrand 2003; Weissmann et al. 2005). In application, distant hybridization and resulting wide hybrids have been widely used as an important tool of chromosome manipulation (also referred to as chromosome engineering) for crop improvement. Based on the chromosome behaviors of wide hybrids and the resulting chromosome constitutions in their progenies, chromosome manipulation of wide hybrids for crop improvement is classified into three main categories:

1. Incorporation of singe-chromosome or chromosome fragment from a wild species (also referred to as alien) into an existing crop in order to enhance crop genetic diversity: The resulting alien chromosome substitution, addition, or translocation lines help breeders to transfer beneficial characteristics from wild and weedy plants to the cultivated crop species. Most efforts to transfer a beneficial trait from wild plants into crops so far have been bridged via alien chromosome translocation lines (Qi et al. 2007).
2. Incorporation of all the alien chromosomes by chromosome doubling in order to produce amphidiploid: Sometimes, the incorporation of partial alien chromosomes will lead to partial amphidiploid. Amphidiploid can be used for the development of a new crop. The man-made crop Triticale (*X Triticosecale* Wittmack) is an amphidiploid between wheat (*Triticum turgidum* L. or *Triticum aestivum* L.) and rye (*Secale cereale* L.) (Gupta and Priyadarshan 1982). Amphidiploid can be further used as a bridge for the development of alien gene introgression or alien chromosome substitution, addition, and translocation lines (Jiang et al. 1994).
3. Elimination of all alien chromosomes in order to induce crop haploid: Haploid is very useful in double-haploid breeding of a true-breeding crop like wheat and rice since it can quickly fix genetic recombination and thus enhance breeding efficiency or facilitate genetic analysis (Pratap et al. 2010).

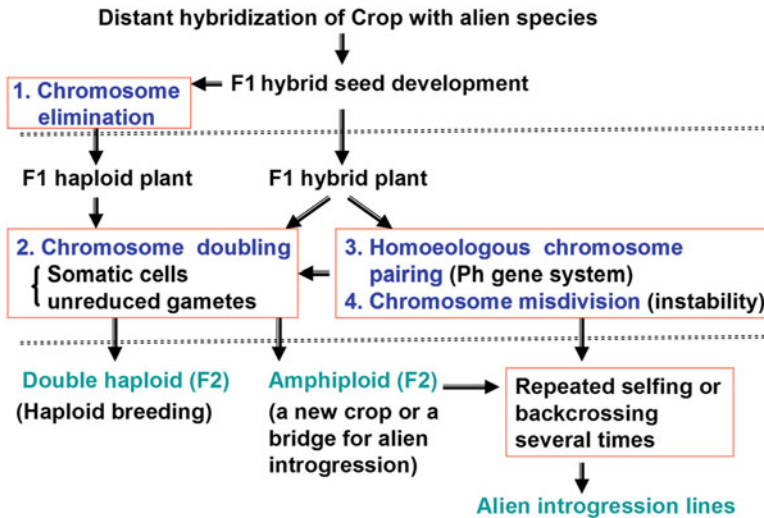

Fig. 2.1 Chromosome manipulation based on chromosome behaviors in F_1 hybrids. Alien chromosome elimination during the development of F_1 hybrid embryos to produce haploid; chromosome doubling in F_1 hybrid plants to produce amphidiploid; homoeologous chromosome pairing or chromosome misdividing in hybrid plants to produce translocation line

Type 1 is the manipulation for single chromosome, while type 2 and 3 are the genome manipulation by the loss and the addition of alien genome, respectively. Chromosome manipulation of wide hybrids for crop improvement is involved in some key steps that may vary according to different wide hybrids. Anyway, a F_1 hybrid between a crop and an alien species is the first step (Fig. 2.1). Crossability of different species is an important genetic character to strongly affect the wide cross. Some genes or QTL for crossability have been found in plants, for example in tribe Triticeae species tetraploid wheat (*T. turgidum* L.) and common wheat (Riley and Chapman 1967; Krowlow 1970; Zheng et al. 1992; Tixier et al. 1998; Liu et al. 1998a, b, 1999, 2002; Alfares et al. 2009; Bertin et al. 2009; Mishina et al. 2009; Zhang et al. 2008a, 2011a). With combined crossable genes/QTL with the application of techniques like embryo rescue and hormone treatment on post-pollination, successful production of F_1 hybrid can be achieved (Jiang et al. 1994; Sharma 1995). In most cases, the production of F_1 hybrid is not a barrier for chromosome manipulation.

2.2 Chromosome Manipulation for Crop Improvement

The chromosome behavior in F_1 hybrid provides us the essential genetic basis of chromosome manipulation. In this review, we focus on the chromosome manipulations based on the chromosome behaviors of wide F_1 hybrids: (1) alien chromosome elimination during the development of F_1 hybrid embryos to produce haploids,

(2) chromosome doubling in somatic cells or gametes in F_1 hybrid plants to produce amphidiploid, and (3) induction of homoeologous pairing in F_1 hybrid plants and then the fixation of translocated chromosomes in their progenies to produce translocation lines (Fig. 2.1).

2.2.1 Chromosome Elimination and Haploid Crop

Chromosome elimination of one parental or uniparental genome after fertilization of the egg by the sperm of another species can occur in intraspecific, interspecific, intergeneric, or more distant hybrids (Dunwell 2010). This phenomenon results in haploid embryo formation of only one of the parents. It is, therefore, a barrier for breeding new crop cultivars by gene introgression from alien species into crop. However, it offers a shortcut for plant breeding by the production of doubled haploids (DH). Plant breeding needs to obtain high levels of homozygous lines with consistent phenotypes. However, the production of such a homozygous line usually requires several generations of selfing or backcrossing even if under the help of molecular markers (Chan 2010). A DH is a homozygous line that can be immediately achieved by artificial (chemical treatment) or spontaneous chromosome doubling of a haploid plant. In fact, doubled-haploid technology can accelerate the breeding of new cultivars, and the time to develop new cultivars may be reduced by 50 % in winter-grown crops compared to classical pedigree breeding (Forster et al. 2007).

Chromosome elimination was observed in interspecific hybridization within a genus like *Nicotiana* (Kostoff 1934; Burk et al. 1979), *Portulaca grandiflora* (Okura 1933), *Hordeum* (Kasha and Kao 1970; Houben et al. 2011), *Solanum* (Uijtewaal et al. 1987; Peloquin et al. 1996; Maine 2003), and *Elymus* (Lu 1992). In genus *Hordeum*, during early development stages of a hybrid embryo of between cultivated barley (*H. vulgare*) as the female and wild species *H. bulbosum*, chromosomes of *H. bulbosum* are eliminated and then lead to the formation of a haploid embryo only containing barley chromosomes. After grown in vitro, a haploid barley plant will be produced. The "*bulbosum*" method was the first haploid induction method to produce large numbers of barley haploids used in crop breeding programs (Choo et al. 1985; Devaux and Pickering 2005). Haploid formation has also been observed in hybrid combination between *H. vulgare* and other species in genus *Hordeum* (Jorgensen and von Bothmer 1988; Houben et al. 2011).

Chromosome elimination has also been shown to exist in many instances of intergeneric or more distant hybridization, such as *Cichorium intybus* × *Cicerbita alpina* (Doré et al. 1996), pear × apple (Inoue et al. 2004), *Brassica napus* × *Orychophragmus violaceus* (Cheng et al. 2002), *B. rapa* × *Isatis indigotica* (Tu et al. 2009), *Avena sativa* × *Zea mays* (Rines and Dahleen 1990; Kynast et al. 2012), *A. sativa* × *Pennisetum americanum* (Matzk 1996), *A. sativa* × Triticeae species (Ishii et al. 2010), *Triticum ventricosum* × *H. bulbosum* (Fedak 1983), *T. turgidum* × *Zea mays* (Almouslem et al. 1998), *T. aestivum* × Triticeae species (*H. vulgare and*

H. bulbosum), and *T. aestivum* × its more distantly related species (*Zea mays*, *Pennisetum glaucum*, *Sorghum bicolor*, *Coix lacryma-jobi*, *Imperata cylindrica*, etc.) (Barclay 1975; Fedak 1980; Laurie and Bennett 1986, 1988; Laurie 1989; Matzk and Mahn 1994; Inagaki and Mujeeb-Kazi 1995; Mochida and Tsujimoto 2001; Gernand et al. 2005; Komeda et al. 2007; Pratap et al. 2005, 2006; Pratap and Chaudhary 2012; Chaudhary et al. 2013). Of them, efficient induction of haploids by hybridization with maize (*Zea mays* L. ssp. *mays*) has been widely reported in wheat (*T. turgidum* and *T. aestivum*), and wheat–maize hybridization is currently a preferred method for producing haploid wheat (Jauhar et al. 2009; Tayeng et al. 2012).

Uniparental chromosome elimination in wide hybrids should be a result of different chromosome behaviors of two parents. There are some assumptions that have been proposed to account for uniparental chromosome elimination in wide hybridization (see review by Houben et al. 2011). One hypothesis is that centromeres from two parents interact unequally with the mitotic spindle, leading to selective chromosome loss (Bennett et al. 1976; Finch 1983; Laurie and Bennett 1989; Kim et al. 2002; Jin et al. 2004; Mochida et al. 2004). Centromeres are the chromosomal loci that attach to spindle microtubules to mediate faithful inheritance of the genome during cell division. They are epigenetically specified by incorporation of the essential kinetochore protein CENH3 (CENP-A in humans or HTR12 in *Arabidopsis thaliana*) (Earnshaw and Rothfield 1985; Talbert et al. 2002), a histone H3 variant that replaces conventional H3 in centromeric nucleosomes (Henikoff and Dalal 2005). The chromosomal location of CENH3 is the assembly site for the kinetochore complex of active centromeres. The loss of CENH3 results in the failure of centromere formation and chromosome segregation (Allshire and Karpen 2008). Recent works on intraspecific (Ravi and Chan 2010) and interspecific hybrids (Sanei et al. 2011) provided the experimental link evidences between the loss of CENH3 and the occurrence of uniparental chromosome elimination. Sanei et al. (2011) studied the mechanism underlying selective elimination of the paternal chromosomes during the early development of *Hordeum vulgare* × *H. bulbosum* embryos and gave the conclusions regarding the role of the centromere-specific histone CENH3 in the process of chromosome elimination: (1) centromere inactivity of *H. bulbosum* chromosomes triggers the mitosis-dependent process of uniparental chromosome elimination in *H. vulgare* × *H. bulbosum* hybrids; (2) centromeric loss of CENH3 protein rather than uniparental silencing of CENH3 genes causes centromere inactivity. They also proposed a possible model of how the mitosis-dependent process of uniparental chromosome elimination works in *H. vulgare* × *H. bulbosum* hybrid embryos. After fertilization, two parental CENH3 genes are transcriptionally active. CENH3 is then loaded into the centromeres of *H. vulgare* but not of *H. bulbosum*, which may be due to cell cycle asynchrony of the two parental genomes during mitotic G2 phase. This leads to *H. bulbosum* chromosome lagging because of centromere inactivity during anaphase, subsequently forming micronuclei. Finally, micronucleated *H. bulbosum* chromatin will degrade, and a haploid *H. vulgare* embryo will develop.

In another experiment, Ravi and Chan (2010) found that haploid *Arabidopsis thaliana* plants can be easily generated through seeds by manipulating the single-centromere protein CENH3. When *cenh3* null mutants expressing altered CENH3 proteins are crossed to wild type, chromosomes from the mutant are eliminated, producing haploid progeny. This process seems a mimic of genome elimination seen in wide hybridization. It is unclear, however, whether a comparable haploidization process takes place between the intraspecific hybrids of *A. thaliana* cenh3-1 null mutants with its wild type (Ravi and Chan 2010) and the interspecific hybrids of *H. vulgare* × *H. bulbosum* (Sanei et al. 2011). From the viewpoints of crop improvement, the production of double haploids has been greatly advanced by the manipulation of CENH3 since the frequency of genome elimination by this kind of centromere-mediated method is higher than any previously reported wide hybridization and thus might improve the efficiency of haploid production in crops (Chan 2010). In addition, crossing a mutant with altered CENH3 proteins (as female) with a wild-type male can shift paternal chromosomes into maternal cytoplasm. This character can be used to develop cytoplasmic male sterility that is very useful for the production of hybrid seeds.

2.2.2 Unreduced Gametes and Amphidiploid

Wide F_1 hybrids from interspecific and intergeneric hybridization usually are amphihaploid with two parental genomes if chromosome elimination does not occur. Due to the absence of only one set of homologous chromosomes, meiosis in F_1 amphihaploid plants (analogous to haploid plant as described above) may result in sterile gametes with incomplete chromosome by meiosis, so there is no seed set. To convert sterile amphihaploids into fertile, duplication of the chromosome complement and then the production of amphidiploids are therefore necessary. Besides restoring fertility, amphidiploids are valuable for alien gene transfer as mentioned in introduction. Chromosome doubling can be carried out through the treatment with anti-microtubule drugs. Colchicine (originally extracted from autumn crocus (*Colchicum autumnale*)) is the most widely used doubling agent although it is highly toxic. This anti-microtubule drug inhibits microtubule polymerization by binding to tubulin. Other doubling agents, such as oryzalin, amiprophosmethyl (APM), trifluralin, and pronamide, all of which are used as herbicides, are also used in the doubling induction with variable degree of success in diploidization.

Some interspecific or intergeneric F_1 amphihaploids can also set grains by selfing and give rise to amphidiploids by spontaneous chromosome doubling. Spontaneous chromosome doubling usually results from unreduced gametogenesis and a union of unreduced female and male gametes leads to the formation of a spontaneous amphidiploid from a wide hybrid. It is believed that unreduced gametes (with somatic chromosome number) played a predominant role in polyploidization (Harlan and De Wet 1975; Ramsey and Schemske 1998, 2002; Cai and Xu 2007; Jauhar 2007; Matsuoka 2011; Silkova et al. 2011), leading to the origination of both

autopolyploids, such as potato (Peloquin et al. 1999), and allopolyploids, such as wheat (Kihara and Lilienfeld 1949). Besides the origin of polyploid species, unreduced gametes can help in crop genetics and breeding, not only in production of amphidiploids in a large scale (Zhang et al. 2010), which is the bridge of alien gene introgression into crop (Yang et al. 2009), but also in the production of doubled haploids (Ramana and Jacobsen 2003; Zhang et al. 2007). Recently, Zhang et al. (2011b) described a simple method for synthesizing DHs (SynDH) especially for allopolyploid species by utilizing unreduced gametes. The method involves three steps: hybridization to induce recombination, interspecific hybridization to extract haploids, and spontaneous chromosome doubling by selfing the interspecific F_1s. SynDHs produced in this way can only contain recombinant chromosomes in the partial genome(s) of interest in a homogeneous background (Zhang et al. 2012; Hao et al. 2013). No special equipment or treatments are involved in the SynDH production and it can be easily applied in any breeding and/or genetic program. Unreduced gametes provide a strategy to fix translocated chromosomes, derived from homoeologous pairing of F_1 hybrids, into their progenies.

Unreduced gametes can be generated by a variety of cytological mechanisms. They are generally formed by anomalies of meiotic cell division in plants. These defects include abnormal spindle orientation, defected synapsis, and omission of chromosome segregation at one of the two meiotic divisions (Veilleux 1985; Bretagnolle and Thompson 1995; Peloquin et al. 1999; Ramana and Jacobsen 2003; Cai et al. 2010; Kynast et al. 2012). The production of unreduced gametes has been largely observed in amphihaploid hybrids of the big tribe Triticeae, including the important polyploid crops like common wheat, durum wheat, barley, rye, triticale (×Triticosecale), and many forage species (for examples, Aase 1930; Kihara and Lilienfeld 1949; Maan and Sasakuma 1977; Islam and Shepherd 1980; Blanco et al. 1983; Fukuda and Sakamoto 1992; Xu and Dong 1992; Li and Liu 1993; Xu and Joppa 1995, 2000; David et al. 2004; Matsuoka and Nasuda 2004; Zhang et al. 2007, 2008b, 2008c, 2010, 2011b, Tiwari et al. 2008; Loureiro et al. 2009; Yang et al. 2010; Matsuoka 2011; Silkova et al. 2011). This haploidy-dependent unreductional meiotic cell division (UMCD), resulting in unreduced gametes, has been considered the mechanism for chromosome doubling in the origins of allopolyploid species in Triticeae and other allopolyploid plant species (Cai and Xu 2007; Jauhar 2007; Matsuoka 2011; Silkova et al. 2011). Two main cytological processes leading to unreductional meiosis were described in *Triticeae* genotypes. First division restitution (FDR) was used to describe the lack of chromosome segregation at anaphase I followed by nuclear restitution and second meiotic division in hybrids between *T. turgidum* L. and *Ae. tauschii* Coss. (Xu and Joppa 2000). On the other hand, also in hybrids of *T. turgidum* with *Ae. tauschii*, other authors described a single-division meiosis (SDM) characterized by a mitosis-like equational division with sister chromatid segregation at anaphase I (Zhang et al. 2007). Both types of divisions, FDR and SDM, may coexist in a same hybrid (Xu and Joppa 2000; Zhang et al. 2007; Silkova et al. 2011; Ressurreição et al. 2012). The divergence in terminology reflects the lack of knowledge regarding the mechanisms for the production of unreduced gametes in amphihaploid genotypes. It should be mentioned that both FDR and

SDM have exactly the same genetic outcome, i.e., formation of two genetically identical unreduced gametes since cell division only involves sister chromatid segregation. That means that no matter which division failed, sister chromatids of a chromosome separated at metaphase of the normal division, like they do in mitosis, leading to formation of unreduced gametes possessing the two non-sister chromatids and consequently retained equivalent levels of parental heterozygosity and epistasis. Zhang et al. (2007), therefore, proposed to call the two mechanism as "mitotic-like meiosis" since it resembles a mitosis by having only one equational division.

Cai et al. (2010) further studied the cytological mechanism of UMCD by using the polyhaploids of wheat cv. Langdon (LDN) and its amphidiploid hybrids with *Aegilops tauschii*. LDN has normal meiosis, but its polyhaploid and amphidiploid have UMCD. They found that sister kinetochores oriented syntelically at meiosis I in LDN but amphitelically in LDN polyhaploid and amphidiploid hybrid. Moreover, sister centromere cohesion persisted until anaphase II in LDN, LDN polyhaploid, and amphidiploid hybrid. Meiocytes with all chromosomes oriented amphitelically underwent UMCD in LDN polyhaploid and amphidiploid, suggesting the tension created by the amphitelic orientation of sister kinetochores, and persistence of centromeric cohesion between sister chromatids at meiosis I contributed to the onset of UMCD. They suggested that some ploidy-regulated genes were responsible for kinetochore orientation at meiosis I in LDN and LDN-derived polyhaploids. In addition, since sister kinetochores of synapsed chromosomes oriented syntelically and asynapsed chromosomes oriented either amphitelically or syntelically, synapsis probably is also a factor for the coordination of kinetochore orientation in LDN. This is agreed with that a high level of chromosome pairing will prevent meiotic restitution and formation of unreduced gametes (Wang et al. 2010), while a low pairing is apparently not sufficient to prevent restitution in interspecific hybrids (Xu and Joppa 2000; Zhang et al. 2008c) or even common wheat itself (Ressurreição et al. 2012). Additionally, in fact, this is also the case observed here for the N5DT5B line under asynaptic conditions. This suggests that processes leading to the previously described haploid-dependent formation of unreduced gametes in F1 hybrids or polyhaploids also occur when two homologous chromosomes are present if synapsis is inhibited.

Second division restitution (SDR): It can also result in unreduced gametes in some dilopploid or hybrids involving autoployploids. SDR was used to describe the normal first meiotic division followed by sister chromatid separation but failed to migrate to opposite poles at meiosis II. Two nuclei with unreduced chromosome number form at the end of meiosis II. Because sister chromatids are kept together in SDR-type gametes, the genetic makeup of these gametes is characterized by high levels of homozygosity, leading to the loss of the majority of parental heterozygosity, and epistatic interaction is lost (Cai and Xu 2007). SDR could occur after the first meiotic division, which should be completely normal, as is the case with meiosis in autopolyploid potato. However, since reductional division and segregation of univalents in anaphase I in wide haploid or amphiploid hybrids do not ensure a balanced chromosome set, SDR is hardly to occur. Therefore, SDR may be not the

pathway for the production of unreduced gametes in wide amphihaploid hybrids as in Trtiticeae.

Production of unreduced gametes in wide hybrids can be stimulated by both genetic (Xu and Joppa 2000; Zhang et al. 2010; Brownfield and Kohler 2011 and the references therein) and environmental (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998; Mable 2004; Pécrix et al. 2011; Mason et al. 2011) factors as well as the interaction between these two (Bretagnolle and Thompson 1995; Zhang et al. 2010; Mason et al. 2011). Genetic studies on mutant alleles responsible for the formation of unreduced gametes in a number of crop species have shown that this phenotype is usually monogenic (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998; Xu and Joppa 2000; Storme and Geelen 2011). Recently, several genes, such as DYAD/SWITCH1 (SWI1), OSD (omission of the second division), CYCA1; 2 (TAM, tardy asynchronous meiosis), AtPSI (Arabidopsis thaliana Parallel Spindles 1), and JASON for unreduced gametes, have been recently identified from the model diploid plant *Arabidopsis thaliana* (Andreuzza and Siddiqi 2008; Ravi et al. 2008; d'Erfurth et al. 2008, 2009; Storme and Geelen 2011). However, the molecular characterization of gene for haploidy-dependent UMCD in wide hybrids has not been reported although this kind of gene is very important for the origin of allopolyploids.

2.2.3 Homoeologous Chromosome Pairing and Translocation Lines

Wild species provides a vast gene pool for crop improvement. Most pioneering efforts in chromosome engineering have involved the *Triticum* species in Triticeae, with the greatest emphasis being placed on improving common wheat (*T. aestivum* L., 2n = 6x = 42, AABBDD) (Qi et al. 2007; Crouch et al. 2009; Reynolds et al. 2009; Wang 2009). Common wheat has more than 300 relative species in the fairly big tribe *Triticeae* (Clayton and Renvoize 1986; Watson and Dallwitz 1994). These species are important resources for broadening the genetic diversity of wheat. Wide hybrids of wheat have been studied since 1876 when Wilson (1876) made the first hybrid between common wheat (*Triticum aestivum* L., 2n = 6x = 42, AABBDD) and rye (*Secale cereale* L., 2n = 2x = 14, RR).

Translocation lines have been recognized as means for providing the most promising pathway for the utilization of alien germplasm (Qi et al. 2007). Translocations can occur in terminal, intercalary, or centric positions. Centric translocations can be produced by the misdivision of univalents at meiosis and subsequent random fusion of telocentric chromosomes, i.e., centric break-fusion (Lukaszewski and Gustafson 1983). In wide hybrids, unpaired chromosomes are present as univalents and thus give a chance to misdivide and then re-fuse. Centric translocation is frequently observed in wide hybrids. However, translocations in other positions are not common since the homoeologous chromosomes between wheat and alien species in wide hybrids show a low pairing level at meiotic metaphase I (MI) due to the action

of pairing homoeologous (*Ph*) gene system in wheat, which restricts the production of wheat–alien translocations (Sears 1976; Martinez-Perez and Moore 2008). This system includes a major pairing gene, *Ph1*, on 5B (Okamoto 1957; Riley and Chapman 1958); an intermediate pairing gene, *Ph2*, on 3D (Mello-Sampayo 1971; Sutton et al. 2003); and several minor loci (Sears 1976). The *Ph1* locus is related to a cluster of genes similar to Cdk2 (cyclin-dependent kinase) in mammals (Griffiths et al. 2006; Al-Kaff et al. 2008; Yousafzai et al. 2010a, b) and has a downstream effect on the synapsis gene *TaASY1* by reducing its expression level (Boden et al. 2009). Cdk2 has been shown to participate in the transition from G1 into S phase and also to affect DNA replication. Although the 5B Cdk-like genes are transcribed, they all seem to be defective copies. These defective Cdk-like genes are responsible for reducing Cdk-type activity, and this leads to the *Ph1* effect. However, the increased Cdk-type activity can phenocopy the effect of deleting the Ph1 locus (Greer et al. 2012). The intermediate pairing gene *Ph2* is involved in the progression of synapsis (Martinez-Perez et al. 2001; Prieto et al. 2004) although the gene responsible for the phenotype is still to be isolated (Sutton et al. 2003). Pairing restriction by *Ph1* and *Ph2* involves not only wheat homoeologues but also wheat–alien chromosomes in wide crosses containing a haploid set of related chromosomes. However, homoeologous chromosomes can pair in hybrids of Chinese Spring (CS) mutant lines *ph1b*, CS*ph2a*, and CS*ph2b* and related alien species enabling gene transfer from alien species to wheat (Wall et al. 1971; Sears 1982; Martinez-Perez and Moore 2008). Moreover, gene Ph^{I} from *Aegilops speltoides* can repress the action of *Ph1* and induce homoeologous chromosome pairing (Chen et al. 1994). A new strategy by increasing Cdk-type activity may be used in the induction of homoeologous chromosome pairing (Greer et al. 2012). The manipulation of *Ph1* gene can relieve the restriction of homoeologous chromosome pairing and thus improve the efficiency of alien translocation development.

On the other hand, natural phenotypic differences in homoeologous pairing have been observed among the hybrids of wheat and alien species (Driscoll and Quinn 1970; Dvorak and McGuire 1981; Farqoo et al. 1990; Ma et al. 1999; Ozkan and Feldman 2001) or in haploids from different common wheat cultivars (Martinez et al. 2005). Of which, Chinese common wheat landrace Kaixian-luohanmai (KL) exhibits homoeologous pairing in hybrids with *Secale cereale* L. (2n = 2x = 14, RR) and *Aegilops variabilis* Eig. (2n = 4x = 28, $UUS^{L}S^{L}$) at levels between those of hybrids involving Chinese Spring *ph1b* (CS*ph1b*) or CS*ph2b*/CS*ph2a* (Luo et al. 1992; Liu et al. 1998c, 2003; Xiang et al. 2005). However, KL × *Psathyrostachys huashanica* Keng ex Kuo (2n = 2x = 14, NsNs) hybrids showed significantly higher chromosome pairing than CS*ph1b* × *Psa. huashanica* (Kang et al. 2008). The lower pairing in CS*ph1b* × *Psa. huashanica* may be caused by a suppressor in *Psa. huashanica* (Sun and Yen 1994). Recently, meiotic phenotypic differences on homoeologous chromosome pairing at metaphase I between hybrids of CS*ph1b* and KL with rye were studied by genomic in situ hybridization (GISH). Although the frequency of wheat–wheat associations was higher in CS*ph1b* × rye than in KL × rye, frequencies of wheat–rye and rye–rye associations were higher in KL × rye than in CS*ph1b* × rye (Hao et al. 2011). These differences may be the result of different mechanisms of control between the ph-like gene(s) controlling

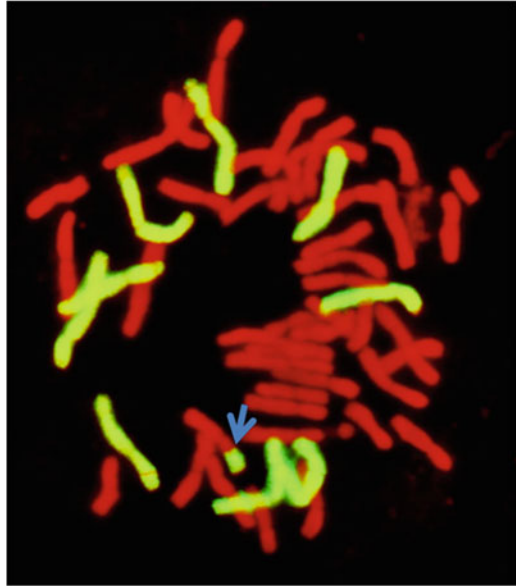

Fig. 2.2 One wheat–rye translocation chromosome (*arrow*) observed at mitotic metaphase in root-tip cells of the Syn-SAU-6/Qinling F_2 plants

homoeologous chromosome pairing in KL and CS*ph1b*. These lines promoting homoeologous chromosome pairing can also be used in alien translocation development.

Another problem for alien translocation development is that randomly separated chromosomes in wide hybrids move towards opposite poles in meiotic anaphase I (AI) and thus result in reduced gametes with absent chromosomes. This sets a barrier for developing translocation lines since the translocated chromosomes may be not contained in reduced gametes or cannot be transmitted into progenies due to the sterility of reduced gametes. High pairing in the inactive *Ph1* gene further reduces fertility of gametes that probably attributed an increase in meiotic disturbances (Ceoloni and Donini 1993). The efficiency of genetic manipulations of *Ph1* gene for the production of wheat–alien translocations in past years was not as good as expected (Miller et al. 1998). How to efficiently transmit the translocated chromosomes induced in F_1 hybrid into following generations still needs to be resolved for translocation line development. As mentioned above, unreduced gametes may be used in the fixation of translocation chromosomes in wide progenies. Translocated chromosomes occurred in meiotic metaphase I in hybrids can be transmissed into amphidiploids by the union of fertile unreduced gametes. Haploid wheat–rye hybrids and its derivatives usually have very low fertility because their reduced gametes are often nonfunctional. However, some synthetic hexaploid wheat Syn-SAU-6/Qinling F_1 plants had relatively high seed set. Syn-SAU-6 was derived from spontaneous chromosome doubling of hybrids between *T. turgidum* L. ssp. *durum* cv. Langdon and *A. tauschii* accession AS65 and inherited the gene(s) for the formation of

unreduced gametes from Langdon (Zhang et al. 2007, 2010). We observed some translocation chromosomes in some F_2 Syn-SAU-6/Qinling hybrid seeds, thus demonstrating that unreduced gametes are capable to fix chromosome rearrangements into progenies. Of which, one wheat–rye translocation chromosome is shown in Fig. 2.2.

References

Aase HC (1930) Cytology of *Triticum, Secale*, and *Aegilops* hybrids with reference to phylogeny. Res Stud State Coll Wash 2:5–60

Alfares W, Bouguennec A, Balfourier F, Gay G, Bergès H, Vautrin S, Sourdille P, Bernard M, Feuillet C (2009) Fine mapping and marker development for the crossability gene *SKr* on chromosome 5BS of hexaploid wheat (*Triticum aestivum* L.). Genetics 183:469–483

Al-Kaff N, Knight E, Bertin I, Foote T, Hart N, Griffiths S, Moore G (2008) Detailed dissection of the chromosomal region containing the *Ph1* locus in wheat *Triticum aestivum*: with deletion mutants and expression profiling. Ann Bot (Lond) 101:863–872

Allshire RC, Karpen GH (2008) Epigenetic regulation of centromeric chromatin: Old dogs, new tricks? Nat Rev Genet 9:923–937

Almouslem AB, Bommineni VR, Jauhar PP, Peterson TS, Rao MB (1998) Haploid durum wheat production via hybridization with maize. Crop Sci 38:1080–1087

Anderson E (1953) Introgressive hybridization. Biol Rev 28:280–307

Andreuzza S, Siddiqi I (2008) Spindle positioning, meiotic nonreduction, and polyploidy in plants. PLoS Genet 4:e1000274

Arnold MA (1997) Natural hybridization and evolution. Oxford University Press, New York

Barclay IR (1975) High frequencies of haploid production in wheat (*Triticum aestivum*) by chromosome elimination. Nature 256:410–411

Bennett MD, Barclay IR, Finch RA (1976) The time rate and mechanism of chromosome elimination in Hordeum hybrids. Chromosoma 54:175–200

Bertin I, Fish L, Foote TN, Knight E, Snape J, Moore G (2009) Development of consistently crossable wheat genotypes for alien wheat gene transfer through fine-mapping of the *Kr1* locus. Theor Appl Genet 119:1371–1381

Blanco A, Simeone R, Tanzarella OA (1983) Morphology and chromosome pairing of a hybrid between *Triticum durum* Desf. and *Haynaldia villosa* (L.) Schur. Theor Appl Genet 64: 333–337

Boden SA, Langridge P, Spangenberg G, Able JA (2009) *TaASY1* promotes homologous chromosome interactions and is affected by deletion of *Ph1*. Plant J 57:487–497

Bretagnolle F, Thompson JD (1995) Gametes with the somatic chromosome number: mechanisms of their formation and role in the evolution of autopolyploid plants. New Phytol 129:1–22

Brownfield L, Kohler C (2011) Unreduced gamete formation in plants: mechanisms and prospects. J Exp Bot 62:1659–1668

Burk LG, Gerstel DU, Wernsman EA (1979) Maternal haploids of *Nicotiana tabacum* L. from seed. Science 206:585

Cai X, Xu SS (2007) Meiosis-driven genome variation in plants. Curr Genomics 8:151–161

Cai X, Xu SS, Zhu XW (2010) Mechanism of haploidy-dependent unreductional meiotic cell division in polyploidy wheat. Chromosoma 119:275–285

Ceoloni C, Donini P (1993) Combining mutations for two homoeologous pairing suppressor genes *Ph1* and *Ph2* in common wheat and in hybrids with alien Triticeae. Genome 36:377–386

Chan SWL (2010) Chromosome engineering: power tools for plant genetics. Trends Biotechnol 28:650–710

Chaudhary HK, Tayeng T, Kaila V, Rather SA (2013) Use of asynchrony in flowering for easy and economical polyhaploid induction in wheat following *Imperata cylindrica*- mediated chromosome elimination approach. Plant Breed. doi:10.1111/pbr.12036

Chen PD, Tsujimoto H, Gill BS (1994) Transfer of *Ph*[I] genes promoting homoeologous pairing from *Triticum speltoides* to common wheat. Theor Appl Genet 88:97–101

Cheng BF, Séguin-Swartz G, Somers DJ (2002) Cytogenetic and molecular characterization of intergeneric hybrids between *Brassica napus* and *Orychophragmus violaceus*. Genome 45:110–115

Choo TM, Reinbergs E, Kasha KJ (1985) Use of haploids in barley breeding. Plant Breed Rev 3:219–252

Clayton WD, Renvoize SA (1986) Genera graminum: grasses of the World. Royal Botanic Garden, Kew

Crouch JH, Payne TS, Dreisigacker S, Wu H, Braun HJ (2009) Improved discovery and utilization of new traits for breeding. In: Dixon J, Braun HJ, Kosina P, Crouch J (eds) Wheat facts and futures. CIMMYT, Mexico, D.F., pp 42–51

David JL, Benavente E, Brès-Patry C, Dusautoir JC, Echaide M (2004) Are neopolyploids a likely route for a transgene walk to the wild? The *Aegilops ovata* × *Triticum turgidum* durum case. Biol J Linn Soc 82:503–510

d'Erfurth I, Jolivet S, Froger N, Catrice O, Novatchkova M, Simon M, Jenczewski E, Mercier R (2008) Mutations in *AtPS1* (*Arabidopsis thaliana* parallel spindle 1) lead to the production of diploid pollen grains. PLoS Genet 4(11):e1000274

d'Erfurth I, Jolivet S, Froger N, Catrice O, Novatchkova M, Mercier R (2009) Turning meiosis into mitosis. PLoS Biol 7(6):e1000124

Devaux P, Pickering RA (2005) Haploids in the improvement of Poaceae. In: Palmer D, Keller W, Kasha KJ (eds) Haploids in crop improvement II. Springer, Heidelberg, Germany, pp 215–242

Doré C, Prigent J, Desprez B (1996) *In situ* gynogenetic haploid plants of chicory (*Cichorium intybus* L.) after intergeneric hybridization with *Cicerbita alpina* Walbr. Plant Cell Rep 15:758–761

Driscoll CJ, Quinn CJ (1970) Genetic variation in *Triticum* affecting the level of chromosome pairing in intergenetic hybrids. Can J Genet Cytol 12:278–282

Dunwell JM (2010) Haploids in flowering plants: origins and exploitation. Plant Biotechnol J 8:377–424

Dvorak J, McGuire PE (1981) Nonstructural chromosome differentiation among wheat cultivars, with special reference to differentiation of chromosomes in related species. Genetics 97: 391–414

Earnshaw WC, Rothfield N (1985) Identification of a family of human centromere proteins using autoimmune sera from patients with Scleroderma. Chromosoma 91:313–321

Ellstrand NC (2003) Dangerous liaisons? When cultivated plants mate with their wild relatives. John Hopkins University Press, Baltimore, MA

Farqoo S, Iqbal N, Shah TM (1990) Intergenetic hybridization for wheat improvement. III. Genetic variation in *Triticum* species affecting homoeologous chromosome pairing. Cereal Res Commun 18:233–237

Fedak G (1980) Production, morphology and meiosis of reciprocal barley-wheat hybrids. Can J Genet Cytol 22:117–123

Fedak G (1983) Haploids in *Triticum ventricosum* via intergeneric hybridization with *Hordeum bulbosum*. Can J Genet Cytol 25:104–106

Finch RA (1983) Tissue-specific elimination of alternative whole parental genomes in one barley hybrid. Chromosoma 88:386–393

Forster BP, Herberle-Bors E, Kasha KJ, Touraev A (2007) The resurgence of haploids in higher plants. Trends Plant Sci 12:368–375

Fukuda K, Sakamoto S (1992) Cytological studies on unreduced male gamete formation in hybrids between tetraploid emmer wheats and *Ae. squarrosa* L. Jpn J Breed 42:255–266

Gernand D, Bruss C, Houben A, Kumlehn J, Matzk F, Prodanovic S, Rubtsova M, Rutten T, Varshney A (2005) Uniparental chromosome elimination at mitosis and interphase in wheat and pearl millet crosses involves micronucleus formation, progressive heterochromatinization, and DNA fragmentation. Plant Cell 17:2431–2438

Greer E, Martín AC, Pendle A, Colas I, Jones AME, Moore G, Shaw P (2012) The Ph1 locus suppresses Cdk2-type activity during premeiosis and meiosis in wheat. Plant Cell 24:152–162

Griffiths S, Sharp R, Foote TN, Bertin I, Wanous M, Reader S, Colas I, Moore G (2006) Molecular characterization of *Ph1* as a major chromosome pairing locus in polyploid wheat. Nature 439:749–752

Gupta PK, Priyadarshan PM (1982) Triticale, present status and future prospects. Adv Genet 21:255–345

Hao M, Luo J, Yang M, Zhang L, Yan Z, Yuan Z, Zheng Y, Zhang H, Liu D (2011) Comparison of homoeologous chromosome pairing between hybrids of wheat genotypes Chinese Spring *ph1b* and Kaixian-luohanmai with rye. Genome 54:959–964

Hao M, Chen J, Zhang L, Luo J, Yuan Z, Yan Z, Zhang B, Chen W, Wei Y, Zhang H, Zheng Y, Liu D (2013) The genetic study utility of a hexaploid wheat DH population with non-recombinant A- and B-genomes. SpringerPlus 2:131

Harlan JR, De Wet JMJ (1975) On Ö. Winge and a prayer: the origins of polyploidy. Bot Rev 41:361–390

Henikoff S, Dalal Y (2005) Centromeric chromatin: what makes it unique? Curr Opin Genet Dev 15:177–184

Houben A, Sanei M, Pickering R (2011) Barley doubled-haploid production by uniparental chromosome elimination. Plant Cell Tiss Org Cult 104:321–327

Inagaki MN, Mujeeb-Kazi A (1995) Comparison of polyhaploid production frequencies in crosses of hexaploid wheat with maize, pearl millet and sorghum. Breed Sci 45:157–161

Inoue E, Sakuma F, Kasumi M, Hara H, Tsukihashi T (2004) Maternal haploidization of Japanese pear through intergeneric hybridization with apple. Acta Hortic 663:815–818

Ishii T, Ueda T, Tanaka H, Tsujimoto H (2010) Chromosome elimination by wide hybridization between Triticeae or oat plant and pearl millet: pearl millet chromosome dynamics in hybrid embryo cells. Chromosome Res 18:821–831

Islam AKMR, Shepherd KW (1980) Meiotic restitution in wheat barley hybrids. Chromosoma 68:252–261

Jauhar PP (2007) Meiotic restitution in wheat polyhaploid (amphihaploids): a potent evolutionary force. J Hered 98:188–193

Jauhar PP, Xu SS, Baenziger PS (2009) Haploidy in cultivated wheats: induction and utility in basic and applied research. Crop Sci 49:737–755

Jiang J, Friebe B, Gill BS (1994) Recent advances in alien gene transfer in wheat. Euphytica 73:199–212

Jin WW, Dawe RK, Henikoff S, Jiang JM, Melo JR, Nagaki K, Talbert PB (2004) Maize centromeres: organization and functional adaptation in the genetic background of oat. Plant Cell 16:571–581

Jorgensen RB, von Bothmer R (1988) Haploids of *Hordeum vulgare* and *H. marinum* from crosses between the two species. Hereditas 108:207–212

Kang HY, Zhang HQ, Wang Y, Jiang Y, Yuan HJ, Zhou YH (2008) Comparative analysis of the homoeologous pairing effects of *phKL* gene in common wheat × *Psathyrostachy huashanica* Keng ex Kuo. Cereal Res Commun 36:429–440

Kasha KJ, Kao KN (1970) High frequency haploid production in barley (*Hordeum vulgare* L.). Nature 225:874–876

Kihara H, Lilienfeld F (1949) A new synthesized 6×wheat. Hereditas suppl:307–319

Kim NS, Armstrong KC, Fedak G, Ho K, Park NI (2002) A microsatellite sequence from the rice blast fungus (Magnaporthe grisea) distinguishes between the centromeres of *Hordeum vulgare* and *H. bulbosum* in hybrid plants. Genome 45:165–174

Komeda N, Chaudhary HK, Suzuki G, Mukai Y (2007) Cytological evidence for chromosome elimination in wheat × *Imperata cylindrica* hybrids. Genes Genet Syst 82:241–248

Kostoff D (1934) A haploid plant of *Nicotiana sylvestris*. Nature 133:949–950

Krowlow KD (1970) Untersuchungen über die Kreuzbarkeit zwischen Weizen und Roggen. Z Pflanzenzücht 64:44–72

Kynast RG, Davis DW, Phillips RL, Rines HW (2012) Gamete formation via meiotic nuclear restitution generates fertile amphiploid F1 (oat × maize) plants. Sex Plant Reprod. doi:10.1007/s00497-012-0182-7

Laurie DA (1989) The frequency of fertilization in wheat × pearl millet crosses. Genome 32:1063–1067

Laurie DA, Bennett MD (1986) Wheat × maize hybridization. Can J Genet Cytol 28:313–316

Laurie DA, Bennett MD (1988) Cytological evidence for fertilization in hexaploid wheat × sorghum crosses. Plant Breed 100:73–82

Laurie DA, Bennett MD (1989) The timing of chromosome elimination in hexaploid wheat × maize crosses. Genome 32:953–961

Li SP, Liu DJ (1993) Cytological analysis on mechanisms of functional gametes formation in *Triticum* hybrids between *Aegilops tauschii* and durum—*Haynaldia villosa* amphidiploid. Acta Genet Sin 20:68–73 (in Chinese with English abstract)

Liu DC, Yen C, Yang JL, Lan XJ, Zheng YL (1998a) The chromosome distribution of crossability gene in durum wheat cv. Langdon. Wheat Inform Serv 87:1–4

Liu DC, Yen C, Yang JL, Zheng YL (1998b) Chromosomal distribution of genes in diploid *Lophopyrum elongatum* (Host) A. Love that influences crossability of wheat with rye. Wheat Inform Serv 86:13–18

Liu DC, Luo MC, Yen C, Yang JL, Yang WY (1998c) The promotion of homoeologous pairing in hybrids of common wheat cv Kaixianluohanmai with alien species. In: Slinkard AE (ed.) Proceedings of ninth international wheat genet symp. University Extension Press, University of Saskatchewan, Saskatoon, Canada, 4. pp. 377–378

Liu DC, Yen C, Yang JL, Zheng YL, Lan XJ (1999) The chromosomal distribution of crossability genes in tetraploid wheat *Triticum turgidum* L. cv. Ailanmai native to Sichuan, China. Euphytica 108:79–82

Liu DC, Lan XJ, Yang ZJ, Zheng YL, Wei YM, Zhou YH (2002) A unique *Aegilops tauschii* genotype needless to embryo rescue in cross with wheat. Acta Bot Sin 44:508–613

Liu DC, Zheng YL, Yan ZH, Zhou YH, Wei YM, Lan XJ (2003) Combination of homoeologous pairing gene *phKL* and *Ph2*-deficiency in common wheat and its meiotic behaviors in hybrids with alien species. Acta Bot Sin 45:1121–1128

Loureiro I, Escorial C, Garcåa-Baudin JM, Chueca MC (2009) Spontaneous wheat—*Aegilops biuncialis*, *Ae. geniculata* and *Ae. triuncialis* amphiploid production, a potential way of gene transference. Span J Agric Res 7:614–620

Lu BR (1992) Dihaploids of *Elymus* from the interspecific crosses *E. dolichatherus* x *E. tibeticus* and *E. brevipes* x *E. panormitanus*. Theor Appl Genet 83:997–1002

Lukaszewski AJ, Gustafson JP (1983) Translocations and modifications of chromosomes in *Triticale* x wheat hybrids. Theor Appl Genet 64:239–248

Luo MC, Yang ZL, Yen C, Yang JL (1992) The cytogenetic investigation on F_1 hybrid of Chinese wheat landrace. In: Ren ZL, Peng JH (eds) Exploration of crop breeding. Science and Technology Press of Sichuan, China, pp 169–176 (in Chinese)

Ma R, Zheng DS, Fan L (1999) The possibility of *ph* genes existing spontaneously in common wheat. Acta Agron Sin 25:99–104 (in Chinese)

Maan SS, Sasakuma T (1977) Fertility of amphihaploids in Triticinae. J Hered 57:76–83

Mable BK (2004) Why polyploidy is rarer in animals than in plants': myths and mechanisms. Biol J Linn Soc 82:453–466

Maine MJ (2003) Potato haploid technologies. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants: a manual. Kluwer Academic Publishers, Dordrecht, pp 241–247

Mallet J (2007) Hybrid speciation. Nature 446:279–283

Martinez M, Cuadrado C, Laurie DA, Romero C (2005) Synaptic behaviour of hexaploid wheat haploids with different effectiveness of the diploidizing mechanism. Cytogenet Genome Res 109:210–214

Martinez-Perez E, Moore G (2008) To check or not to check? The application of meiotic studies to plant breeding. Curr Opin Plant Biol 11:222–227

Martinez-Perez E, Shaw P, Moore G (2001) The *Ph1* locus is needed to ensure specific somatic and meiotic centromere association. Nature 411:204–207

Mason AS, Nelson MN, Yan G, Cowling WA (2011) Production of viable male unreduced gametes in *Brassica* interspecific hybrids is genotype specific and stimulated by cold temperatures. BMC Plant Biol 11:103

Matsuoka Y (2011) Evolution of polyploidy *Triticum* wheats under cultivation: the role of domestication, natural hybridization and allopolyploid speciation in their diversification. Plant Cell Physiol 52:750–764

Matsuoka Y, Nasuda S (2004) Durum wheat as a candidate for the unknown female progenitor of bread wheat: an empirical study with a highly fertile F_1 hybrid with *Aegilops tauschii* Coss. Theor Appl Genet 109:1710–1717

Matzk F (1996) Hybrids of crosses between oat and andropogoneae or paniceae species. Crop Sci 36:17–21

Matzk F, Mahn A (1994) Improved techniques for haploid production in wheat using chromosome elimination. Plant Breed 113:125–129

Mello-Sampayo T (1971) Genetic regulation of meiotic chromosome pairing by chromosome 3D of *Triticum aestivum*. Nature New Biol 230:22–23

Miller TE, Reader SM, Shaw PJ, Moore G (1998) Towards an understanding of the biological action of the *Ph1* locus in wheat. In: Slinkard AE (ed.) Proceedings of the ninth international wheat genetic symposium. University Extension Press, Saskatoon. pp.17–19

Mishina K, Sato H, Manickavelu A, Sassa H, Koba T (2009) Molecular mapping of *SKr* for cross-ability in common wheat. Breed Sci 59:679–684

Mochida K, Tsujimoto H (2001) Production of wheat double haploids by pollination with Job's Tears (*Coix lacrymajobi* L.). Heredity 92:81–83

Mochida K, Sasakuma T, Tsujimoto H (2004) Confocal analysis of chromosome behavior in wheat × maize zygotes. Genome 47:199–205

Okamoto M (1957) Asynapsis effect of chromosome V. Wheat Inform Serv 5:6

Okura E (1933) A haploid plant in *Portulacea grandiflora* Hook. Jpn J Genet 8:251–260

Ozkan H, Feldman M (2001) Genotypic variation in tetraploid wheat affecting homoeologous pairing in hybrids with *Aegilops peregrine*. Genome 44:1000–1006

Pécrix Y, Rallo G, Folzer H, Cigna M, Gudin S, Le Bris M (2011) Polyploidization mechanisms: temperature environment can induce diploid gamete formation in *Rosa* sp. J Exp Bot 62:3587–3597

Peloquin SJ, Boiteux LS, Carputo D (1999) Meiotic mutants in potato: valuable variants. Genetics 153:1493–1499

Peloquin SJ, Gabert AC, Ortiz R (1996) Nature of 'pollinator' effect in potato (*Solanum tuberosum* L.) haploid production. Ann Bot 77:539–542

Pratap A, Sethi GS, Chaudhary HK (2005) Relative efficiency of different Gramineae genera for haploid induction in triticale and triticale × wheat hybrids through the chromosome elimination technique. Plant Breed 124:147–153

Pratap A, Sethi GS, Chaudhary HK (2006) Relative efficiency of anther culture and chromosome elimination techniques for haploid induction in triticale × wheat and triticale × triticale hybrids. Euphytica 150:339–345

Pratap A, Choudhary AK, Kumar J (2010) *In vitro* techniques towards genetic enhancement of food legumes—a review. J Food Legumes 23:169–185

Pratap A, Chaudhary HK (2012) Effect of auxins on induction of polyhaploids in triticale and triticale × wheat hybrids through the chromosome elimination technique. Indian J Agric Sci 82:66–70

Prieto P, Shaw P, Moore G (2004) Homologue recognition during meiosis is associated with a change in chromatin structure. Nat Cell Biol 6:906–908
Qi L, Friebe B, Zhang P, Gill BS (2007) Homoeologous recombination, chromosome engineering and crop improvement. Chromosome Res 15:3–19
Ramana MS, Jacobsen E (2003) Relevance of sexual polyploidization for crop improvement—a review. Euphytica 133:3–18
Ramsey J, Schemske DW (1998) Pathways, mechanisms and rates of polyploid formation in flowering plants. Annu Rev Ecol Syst 29:467–501
Ramsey J, Schemske DW (2002) Neopolyploidy in flowering plants. Annu Rev Ecol Syst 33:589–639
Ravi M, Chan SWL (2010) Haploid plants produced by centreomere-mediated genome elimination. Nature 464:615–619
Ravi M, Marimuthu MPA, Siddiqi I (2008) Gamete formation without meiosis in *Arabidopsis*. Nature 451:1121–U1110
Ressurreição F, Barão A, Viegas W, Delgado M (2012) Haploid independent unreductional meiosis in hexaploid wheat. In: Swan A (ed) Meiosis - molecular mechanisms and cytogenetic diversity. Tech Press, Dublin, pp 321–330
Reynolds M, Foulkes MJ, Slafer GA, Berry P, Parry MA, Snape JW, Angus WJ (2009) Raising yield potential in wheat. J Exp Bot 60:1899–1918
Riley R, Chapman V (1958) Genetic control of the cytologically diploid behaviour of hexaploid wheat. Nature 13:713–715
Riley R, Chapman V (1967) The inheritance in wheat of crossability with rye. Genet Res 9:259–267
Rines HW, Dahleen LS (1990) Haploid oat plants produced by application of maize pollen to emasculated oat florets. Crop Sci 30:1073–1078
Sanei M, Pickering R, Kumke K, Nasuda S, Houben A (2011) Loss of centromeric histone H3 (CENH3) from centromeres precedes uniparental chromosome elimination in interspecific barley hybrids. Proc Natl Acad Sci U S A 108:13373–13374
Sears ER (1976) Genetic control of chromosome pairing in wheat. Annu Rev Genet 10:31–51
Sears ER (1982) A wheat mutant conditioning an intermediate level of homoeologous pairing. Can J Genet Cytol 24:715–719
Silkova OG, Shchapova AI, Shumny VK (2011) Meiotic restitution in amphihaploids in the tribe Triticeae. Russ J Genet 47:383–393
Sharma HC (1995) How wide can a wide cross be? Euphytica 82:43–64
Stebbins GL (1971) Chromosomal evolution in higher plants. Addison-Wesley, Reading, MA
Storme ND, Geelen D (2011) The Arabidopsis mutant *jason* produces unreduced FDR male gametes through a parallel/fused spindle mechanism in meiosis II. Plant Physiol 155:1403–1415
Sun GL, Yen C (1994) The ineffectiveness of the *ph1b* gene on chromosome association in the F_1 hybrid *Triticum aestivum* × *Psathyrostachys huashanica*. Wheat Inform Serv 79:28–32
Sutton T, Whitford R, Baumann U, Dong CM, Able JA, Langridge P (2003) The Ph2 pairing homoeologous locus of wheat (*Triticum aestivum*): identification of candidate meiotic genes using a comparative genetics approach. Plant J 36:443–456
Tayeng T, Chaudhary HK, Kishore N (2012) Enhancing doubled haploid production efficiency in wheat (*Triticum aestivum* L. em. Thell) by *in vivo* colchicine manipulations in *Imperata cylindrica-mediated* chromosome elimination approach. Plant Breed. doi:10.1111/j.1439-0523.2012.01986.x
Talbert PB, Masuelli R, Tyagi AP, Comai L, Henikoff S (2002) Centromeric localization and adaptive evolution of an *Arabidopsis* histone H3 variant. Plant Cell 14:1053–1066
Tixier MH, Sourdille P, Charmet G, Gay G, Jaby C, Cadalen T, Bernard S, Nicolas P, Bernard M (1998) Detection of QTLs for crossability in wheat using a doubled-haploid population. Theor Appl Genet 97:1076–1082
Tiwari VK, Rawat N, Neelam K, Randhawa GS, Singh K, Chhuneja P, Dhaliwal HS (2008) Development of *Triticum turgidum* subsp. *durum*—*Aegilops longissima* amphiploids with high iron and zinc content through unreduced gamete formation in F_1 hybrids. Genome 51:757–766
Tu YQ, Sun J, Ge XH, Li ZY (2009) Chromosome elimination, addition and introgression in intertribal partial hybrids between *Brassica rapa* and *Isatis indigotica*. Ann Bot 103:1039–1048

Uijtewaal BA, Huigen DJ, Hermsen JGT (1987) Production of potato monohaploids (2n = x = 12) through prickle pollination. Theor Appl Genet 73:751–758

Veilleux R (1985) Diploid and polyploid gametes in crop plants: mechanisms of formation and utilization in plant breeding. Plant Breed Rev 3:252–288

Wall AM, Riley R, Chapman V (1971) Wheat mutants permitting homoeologous meiotic chromosome pairing. Genet Res 18:311–328

Wang CJ, Zhang LQ, Dai SF, Zheng YL, Zhang HG, Liu DC (2010) Formation of unreduced gametes is impeded by homologous chromosome pairing in tetraploid *Triticum turgidum* × *Aegilops tauschii* hybrids. Euphytica 175:323–329

Wang DW (2009) Wide hybridization: engineering the next leap in wheat yield. J Genet Genomics 36:509–510

Watson L, Dallwitz MJ (1994) The Grass Genera of the World, 2nd edn. CAB International, Oxforshire, UK

Weissmann S, Feldman M, Gressel J (2005) Sequence evidence for sporadic intergeneric DNA introgression from wheat into a wild *Aegilops* species. Mol Biol Evol 22:2055–2062

Wilson AS (1876) On wheat and rye hybrids. Trans Proc Bot Soc Edinburgh 12:286–288

Xiang ZG, Liu DC, Zheng YL, Zhang LQ, Yan ZH (2005) The effect of *phKL* gene on homoeologous pairing of wheat-alien hybrids is situated between gene mutants of *Phl* and *Ph2*. Hereditas (Beijing) 27:935–940 (in Chinese)

Xu SJ, Dong YS (1992) Fertility and meiotic mechanisms of hybrids between chromosome autoduplication tetraploid wheats and *Aegilops* species. Genome 35:379–384

Xu SJ, Joppa LR (1995) Mechanisms and inheritance of first division restitution in hybrids of wheat, rye, and *Aegilops squarrosa*. Genome 38:607–615

Xu SJ, Joppa LR (2000) First division restitution in hybrids of Langdon durum disomic substitution lines with rye and *Aegilops squarrosa*. Plant Breed 119:233–241

Yang WY, Liu DC, Li J, Zhang LQ, Wei HT, Hu XR, Zheng YL, He ZH, Zou YC (2009) Synthetic hexaploid wheat and its utilization for wheat genetic improvement in China. J Genet Genomics 36:539–546

Yang YW, Zhang LQ, Yen Y, Zheng YL, Liu DC (2010) Cytological evidence on meiotic restitution in pentaploid F_1 hybrids between synthetic hexaploid wheat and *Aegilops variabilis*. Caryologia 63:354–358

Yousafzai FK, Al-Kaff N, Moore G (2010a) Structural and functional relationship between the *Ph1* locus protein 5B2 in wheat and CDK2 in mammals. Funct Integr Genomics 10:157–166

Yousafzai FK, Al-Kaff N, Moore G (2010b) The molecular features of chromosome pairing at meiosis: the polyploid challenge using wheat as a reference. Funct Integr Genomics 10:147–156

Zhang L, Wang J, Zhou R, Jia J (2011a) Discovery of quantitative trait loci for crossability from a synthetic wheat genotype. J Genet Genomics 38:373–378

Zhang L, Luo J, Hao M, Zhang L, Yuan Z, Yan Z, Liu Y, Zhang B, Liu B, Liu C, Zhang H, Zheng Y, Liu D (2012) Genetic map of *Triticum turgidum* based on a hexaploid wheat population without genetic recombination for D genome. BMC Genet 13:69

Zhang LQ, Yen Y, Zheng YL, Liu DC (2007) Meiotic restriction in emmer wheat is controlled by one or more nuclear genes that continue to function in derived lines. Sex Plant Reprod 20:159–166

Zhang LQ, Yan ZH, Dai SF, Chen QJ, Yuan ZW, Zheng YL, Liu DC (2008a) The crossability of *Triticum turgidum* with *Aegilops tauschii*. Cereal Res Commun 37:417–427

Zhang LQ, Liu DC, Lan XJ, Zheng YL, Yan ZH (2008b) A synthetic wheat with 56 chromosomes derived from *Triticum turgidum* and *Aegilops tauschii*. J Appl Genet 49:41–44

Zhang LQ, Chen QJ, Yuan ZW, Xiang ZG, Zheng YL, Liu DC (2008c) Production of aneuhaploid and euhaploid sporocytes by meiotic restitution in fertile hybrids between durum wheat Langdon chromosome substitution lines and *Aegilops tauschii*. J Genet Genomics 35:617–623

Chapter 3
Tissue Culture and Regeneration: A Prerequisite for Alien Gene Transfer

Maria Wędzony, Magdalena Szechyńska-Hebda, Iwona Żur, Ewa Dubas, and Monika Krzewska

Abstract Introgression of genes from alien species into crop plants could be achieved through distant hybridisation aided by tissue culture-based embryo rescue techniques. Beside this, in vitro mutagenesis, gametoclonal/somaclonal variation and transgenesis are the other tools which can generate additional variability. However, all these tissue culture-based tools require totipotent tissues. The direct regeneration of plants from an explant without a callus stage via organogenesis or somatic embryogenesis is the quickest path for micropropagation. Because of their speed and low costs of culture phase and the fidelity of the genotype in the cloned progeny, systems with direct somatic embryogenesis or organogenesis are often recommended and subjected to transformation. On the other hand, most micropropagation procedures with a callus stage can be applied as a basis for transformation, and the fresh friable calli can be directly used as the transformation target. Cell and microspore suspension cultures have also been seen as the ideal targets for genetic transformation due to the large amount of homogenous material, easy selection of the targeted cells and less chances of chimeric regeneration, while protoplasts due to exposed plasma membrane can introduce foreign DNA very easily and therefore form the ideal targets for generating unique and novel plants. This chapter discusses various plant regeneration methods and the factors affecting them towards achieving alien gene transfer in crop plants.

Keywords Callus • Embryogenesis • In vitro • Protoplasts • Somatic organogenesis • Suspension culture • Transformation

---

M. Wędzony, Ph.D. (✉)
Pedagogical University of Krakow, Podchorążych 2, 30-084 Krakow, Poland
e-mail: mwedzony@ap.krakow.pl

M. Szechyńska-Hebda • I. Żur • E. Dubas • M. Krzewska
Institute of Plant Physiology, Polish Academy of Sciences, Niezapominajek 21, 30-239 Krakow, Poland

A. Pratap and J. Kumar (eds.), *Alien Gene Transfer in Crop Plants, Volume 1: Innovations, Methods and Risk Assessment*, DOI 10.1007/978-1-4614-8585-8_3,
© Springer Science+Business Media New York 2014

Abbreviations

2,4-D	2,4-Dichlorophenoxy acetic acid
BA	6-Benzyladenine
BAP	Benzylaminopurine
IAA	Indole-3-acetic acid
PAA	Phenyl acetic acid
IBA	Indole-3-butyric acid
PGR	Plant growth regulators
SE	Somatic embryo
WPM	Woody plant medium
CPPU	*N*-(2-Chloro-4-pyridyl)-*N′*-phenylurea
MS	Basal medium according to Murashige and Skoog (1962)
B5	Basal medium according to Gamborg Miller and Ojima (1968)
NAA	1-Naphthalene acetic acid
TDZ	Thidiazuron

3.1 Introduction

The plant genetic transformation process can usually be divided into the following phases: (1) explant selection and preparation; (2) transfer of foreign DNA to the explant genome; (3) selection of the transformed cell/tissue and its regeneration into a plant; (4) confirmation of the transgenic nature of the regenerated plant, including stabilisation of the transgene at the homozygotic level and (5) analysis of transgene expression. In most of the protocols, first three steps of plant transformation are performed in vitro. Therefore, the factors influencing transformation effectiveness are directly related to the specific requirements of the in vitro culture. These key factors include the quality and developmental stage of the explants at the time of culture initiation, the composition of the culture medium and the culture conditions prior to and after gene transfer, treatment of the culture to reduce tissue damage during gene transfer and the specific parameters of both the gene transfer and selection systems (Popelka and Altpeter 2003). All of these factors can hinder the efficiency of a transformation protocol, unless they are optimised. High-throughput transformation systems use a target tissue that can easily be isolated and processed through the in vitro protocol with a high regeneration capacity. Therefore, various culture protocols are optimised depending on the genetic background of the plant being subjected to the transformation. This chapter describes the different plant regeneration systems.

3.2 In Vitro Plant Regeneration

The main plant regeneration systems can be divided based on the tissue targeted for the gene transfer: (1) explant cultures without callusing with subsequent direct organogenesis and/or somatic embryogenesis; (2) tissue callusing with subsequent

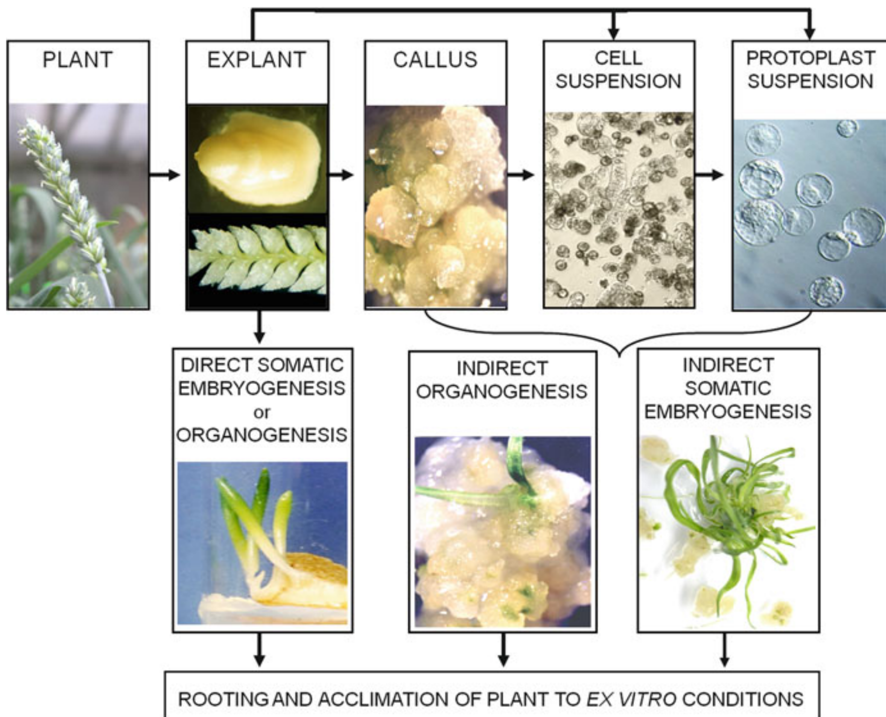

Fig. 3.1 Main in vitro-cultured targets for transformation and transgenic plant regeneration pathways. *Explants*: Isolated and cultured young plant organs or tissues (apical shoot meristems, young leaves, hypocotyls, embryos, stem pieces and others) used directly for transformation, further regenerated to plant via direct organogenesis or somatic embryogenesis without the callus phase. *Callus*: Initiated from explants, an unorganised mass of cells, transformed and regenerated through indirect organogenesis or somatic embryogenesis. *Cell suspension*: Prepared by transferring young plant tissue or callus to a liquid medium and cells or cell clumps released by agitation. After transformation, plant regeneration is performed similarly to the callus cultures. *Protoplast suspension*: Plant cells in a suspension devoid of a cell wall by enzymatic digestion. After transformation and removal of the wall-digesting enzymes, a new cell wall formation leads to the establishment of a cell suspension culture, and the regeneration steps are performed accordingly. *Organogenesis*: A formation of plant organs (shoots or roots) from meristematic cell clumps induced on a surface or within cultured intact explants or callus. *Somatic embryogenesis*: The process by which an embryo-like structure is formed from a single somatic cell of an explant or a callus. Somatic embryos develop into plantlets in a way resembling zygotic embryogenesis. Organogenesis or somatic embryogenesis is called "*direct*" when the process does not involve callus formation or *"indirect"* when organs or somatic embryos are formed via callus tissue derived from the explants or on the surface of already in vitro-regenerated organs

indirect organogenesis and/or somatic embryogenesis; (3) cell suspension culture and (4) protoplast culture (Fig. 3.1). These systems are described in the following separate sections.

3.2.1 *Plant Regeneration via Direct Organogenesis or Somatic Embryogenesis*

3.2.1.1 Nature of the Process

The direct regeneration of plants from various explants of plant tissue without a callus stage is the quickest path for micropropagation. These types of tissue culture protocols were among the first to be established and they are well described in manuals (Street 1973; Reinert and Yeoman 1982; Bhojwani and Razdan 1992; Dirr and Heuser 2009; Smith 2012). The process of direct regeneration of plants can happen in two ways: via organogenesis or somatic embryogenesis. The type of regeneration pathway mainly depends on the composition of the plant growth regulators (PGRs) which has been, among others, clearly demonstrated in the culture of leaf discs of sandal wood (*Santalum album*) (Bele et al. 2012), the immature embryonic stalks of *Bixa orellana* (Sharon et al. 2012) or in various chickpea (*Cicer arietinum*) explants on a variety of induction media differing exclusively in their PGR composition (Anwar et al. 2010). In vitro organogenesis is the process of plant organ formation and is called “direct” when the shoots or the roots are formed by groups of explant cells without a phase of callus formation. In order to regenerate the plants, the shoots are usually detached from the explants and transferred to a new medium to be rooted. Then the new plants can be potted in ex vitro conditions, and in this way the whole micropropagation cycle is accomplished. In vitro-induced roots usually do not grow into plants, and in this sense this is a dead end of the micropropagation protocol. However, a hairy root culture is often the goal when the culture is performed in order to obtain secondary metabolites in vitro, for example: flavonoids (Zhang et al. 2009), resveratrol (Kim et al. 2008) or caffeic acid derivatives (Stojakowska et al. 2012). Therefore, many protocols of this kind are developed, including the transformation of such culture with the purpose of obtaining a higher concentration of the desired metabolite as reviewed by Cai et al. (2012).

Direct somatic embryogenesis is a process in which a single cell of the explant forms an embryo-like structure that mimics the development of zygotic embryos. The induction medium provides the proper nutrition and PGRs. Somatic embryos (SEs) usually germinate into plants when transferred to germination media. Both in vitro-obtained shoots and SEs can be subcultured in vitro on induction media to raise the effect of material multiplication; however, the effect of speed is lost this way.

3.2.1.2 Explants and Media

The variability of explants has recently been used to obtain direct somatic embryogenesis (Loganathan et al. 2010; Piqueras et al. 2010; Aboshama 2011; Nada et al. 2011; Banerjee et al. 2012; Bele et al. 2012; Bhagyawant et al. 2012; Patil and Paikrao 2012; Xi et al. 2012; Zhao et al. 2012a, b) or shoot formation

(Siddique and Anis 2009; Alam et al. 2010; Borna et al. 2010; Purkayastha et al. 2010; Kurmi et al. 2011; Dey et al. 2012; Kumar and Bhat 2012; Park et al. 2012). Among the most typical are the fragments of young seedlings: cotyledons, cotyledonary nodes, hypocotyls and immature embryonic shoot tips. Another group of explants are leaves or leaf discs, petioles, shoot tips and nodal or inter-modal stem segments. SEs have also recently been obtained from young root discs cultured in a liquid induction medium (Yang et al. 2010). In recent years, most studies have been based on jellified Murashige and Skoog (MS) basal medium, sometimes combined with Gamborg's B5 vitamins. In a few cases, a positive effect was achieved on liquid media (Yang et al. 2010; Aviles-Vinas et al. 2012). However, other types of basic media could also be an effective base of the protocol, as shown by the culture of *Piper nigrum* shoot tips and the nodal segments on Schenk and Hildebrandt medium (Schenk and Hildebrandt 1972; Maju and Soniya 2012) or the culture of the shoots and nodal explants of *Tribulus terrestris* (Raghu et al. 2010) or *Capsicum annuum* (Aboshama 2011) on woody plant medium (WPM) (Lloyd and McCown 1980).

A variety of PGRs are in use to induce direct somatic embryogenesis or organogenesis. Among them are auxins or auxin analogues, alone (for example: Loganathan et al. 2010; Yang et al. 2010; Aviles-Vinas et al. 2012; Banerjee et al. 2012; Bhagyawant et al. 2012) or in combination with thidiazuron (TDZ), kinetine and/or giberelin (Siddique and Anis 2009; Anwar et al. 2010; Borna et al. 2010; Purkayastha et al. 2010; Aboshama 2011; Parimalan et al. 2011; Zhao et al. 2012a). It is worth to notice some original compounds applied for direct SE or shoot induction. Zhao et al. (2012b) found positive effect of CPPU on induction of somatic embryos on petiole explants of *Epipremnum aureum*. Park et al. (2012) raised the number of induced shoots and their growth rate by addition of silver nitrate and putrescine to the induction medium. Greer et al. (2009) showed that modification of the ammonium nitrate content in the direct somatic embryogenesis induction medium can increase the number of primary embryos produced over twofold in the elite hard red wheat cultivar "Superb".

Somatic embryo germination is usually achieved on hormone-free media and/or with half-strength salt concentration. Sometimes, PGRs have been helpful. For instance, low concentration of BAP (Banerjee et al. 2012) supported *Bauhinia variegata* SE germination. Dessication of soybean (*Glycine max*) SEs on sterile Petri dishes, prior to their germination on hormone-free MS medium was successfully applied by Loganathan et al. (2010). In contrast, shoot rooting often requires addition of PGR. Alam et al. (2010) raised rooting efficiency in *Ricinus communis* by applying combination of BAP with GA_3, while BAP with IAA was applied in *Solanum tuberosum* for the same purpose (Borna et al. 2010). Similarly, IBA enabled rooting in *Piper nigrum* (Maju and Soniya 2012) and *Vitis vinifera* (Kurmi et al. 2011).

3.2.1.3 Genome Stability and Transformation

One of the most desirable effects of direct and quick plant regeneration is fidelity of the genotype in the cloned progeny. Genome stability was confirmed, among others,

by DNA marker analysis in sugarcane (*Saccharum officinarum*) plants, regenerated from young whorl leaf (Pandey et al. 2012), and patchouli (*Pogostemon cablin*), regenerated from mature leaf discs (Paul et al. 2010).

Because of their speed and low costs of culture phase, the systems with direct somatic embryogenesis or organogenesis are often recommended and/or subjected to transformation. Both of these well-established methods in plant transformation are used in combination with direct regeneration protocols: particle bombardment, also known as "biolistic method" (Greer et al. 2009; Purkayastha et al. 2010; Taparia et al. 2012), and the *Agrobacterium* method (Borna et al. 2010; Parimalan, et al. 2011; Dey et al. 2012; Maju and Soniya 2012; Sujatha et al. 2012). In some studies *Agrobacterium rhizogenes* was also used (Yoshimatsu 2008). The transformation methods are essentially the same as those used in callus culture and are described in more detail in the next section. Interestingly, treatment with particles or *Agrobacterium* can be applied at various phases of the protocol, e.g. at its very start, before the effects of shoots and/or SEs are visible (Sujatha et al. 2012) or when juvenile structures covered by thin cell walls appear (Borna et al. 2010; Parimalan, et al. 2011; Dey et al. 2012; Maju and Soniya 2012). A decision should then be taken considering the efficiency of transformation and regeneration.

The direct regeneration of shoots or SE induction processes is separated from callusing by the thin balance of PGR composition. Taparia et al. (2012) have recently compared the efficiency of the transformation of SEs obtained in sugarcane via both direct and indirect regeneration processes with the biolistic method. They found higher transformation efficiency with indirectly regenerated SEs, while the transformation of the direct SEs substantially shortened the process.

3.2.2 Plant Regeneration via Callus Culture

Most micropropagation procedures with a callus stage can be applied as a basis for transformation. The typical protocol stages involve explant isolation and disinfection and then callus induction followed by plant regeneration. Freshly collected explants before callusing or fresh friable calli are most often used as the transformation targets. The callus propagation stage is reduced to minimum to prevent the high incidence of somaclonal variation and chimerism. Plants are regenerated by either of the two pathways: organogenesis or somatic embryogenesis. In the process of organogenesis, a shoot or a root differentiated from a group of cells is tightly connected by the procambial strands with the explant or the callus, whereas the somatic embryo develops from a single cell and has no vascular connection with the tissue from which it originated. Organogenic differentiation of the so-called organ primordia leads to the formation of a cluster of cambium-like cells (meristemoid), which develops into an organ in a monopolar manner. Due to the initial meristemoid plasticity, both roots and shoots can be regenerated (Jimenez 2001).

3.2.2.1 Donor Plant Effect

The efficiency of callus formation is strongly affected by the genotype of the explant donor. Protocol improvements made by adding or changing the concentration of various media components or altering the culture conditions should always be referred to the genotype under investigation. In barley, for example, Bregitzer and Campbell (2001) and Tyagi et al. (2010) described the identification of QTL associated with green plant regeneration. These loci included a ferredoxin–nitrite reductase (NIR) gene linked to high regeneration ability (Nishimura et al. 2005) and genes involved in a hormonal response known to influence plant regeneration (Jha et al. 2007; Harwood 2012).

The genotypic effect is modified by the vitality of the donor plant and its physiological stage (Szechyńska-Hebda et al. 2012). The latter is under the influence of the plant environment; therefore, different results can be expected from the same genotype grown under field or glasshouse conditions, under optimal light and irrigation or subjected to physiological stress (Mitić et al. 2009).

The cells of explants suitable for callusing and regeneration should be totipotent. Totipotency is predetermined and maintained through many generations of cells cultured in vitro; thus, the competent culture might retain the "memory" of the capacity of the initial cells (Szechyńska-Hebda et al. 2012). However, totipotent calli can lose their regeneration capacity when they are subjected to too many subculture cycles. Generally, competent calli preferentially contain small cells with a dense cytoplasm and compact nuclei, and they seem morphologically younger in comparison to the large, differentiated, highly vacuolated cells of the non-competent calluses (Street 1973; Reinert and Yeoman 1982; Bhojwani and Razdan 1992; Smith 2012; Szechyńska-Hebda et al. 2012). The maintenance of small cell volumes is believed to be one of the conditions needed to enable the onset of embryogenesis/organogenesis (Pellegrineschi et al. 2004) and to represent a dedifferentiated cell state with the potency to initiate a new developmental programme (Pasternak et al. 2002). Several pretreatments or culture conditions can support totipotency and regeneration efficiency in competent tissue; however, they cannot break the internal barriers against induction of the regeneration process if the tissue is non-competent (Szechyńska-Hebda et al. 2007, 2012).

3.2.2.2 Media in Callus Culture

A range of culture media components have been evaluated or the levels of the key nutrients manipulated in order to enhance regeneration in different culture systems. PGRs play a key role in callus culture protocols (Street 1973; Reinert and Yeoman 1982, Bhojwani and Razdan 1992; Smith 2012). For instance, it was shown in *Arabidopsis* that the organogenesis pathway depends on a high cytokinin:auxin ratio (Zuo et al. 2002), while the high cytokinin level promotes *WUS* expression, which is essential to induce the de novo formation of the shoot meristem (Su et al. 2011).

In contrast, the formation of somatic embryos in wheat requires a high concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) (Szechyńska-Hebda et al. 2007). Picloram and TDZ were shown to act as powerful growth regulators in cereals (Schulze 2007; Barro et al. 1999; Ganeshan et al. 2003). He et al. (2010) found that the concentration of picloram increased from 2 to 10 mg/l and resulted in an improved final transformation frequency from 2.8 to 6.3 %. The regenerated leaf tissues of dicotyledonous species, e.g. sunflower, strawberry or tobacco, are often maintained in a medium supplemented with TDZ (0.1–2.0 mg/l) or isopentenyladenine (2iP) (2.0 mg/l) and indol-3 butyric acid (IBA), α-naphthalene acetic acid (NAA) or indole-3-acetic acid (IAA) (each 0.5 mg/l) (Silva 2005; Hanhineva and Kärenlampi 2007; Sujatha et al. 2012). The callus stage of potato is facilitated by treating the explant with zeatin or zeatin riboside in combination with low levels of NAA or IAA. Other growth regulators that have been successfully used in combination for callus induction and growth are kinetin, 2,4-D and gibberellic acid (Turhan 2004). The stage corresponding to shoot production preferably has the cytokinin and auxin level reduced as well as the addition of gibberellin to stimulate shoot elongation (Heeres et al. 2002). The above examples are only a narrow probe of various growth regulator applications.

Besides the growth regulators, some other media components may substantially influence the culture effect. Lipoic acid, an antioxidant, was effective in increasing the number of responding embryogenic calluses in wheat transformation experiments (Dan et al. 2009). Bartlett et al. (2008) developed a high-throughput transformation system for barley, and during its optimisation, enhancement was achieved by elevating the copper concentration in the culture media. This doubled the number of regenerated shoots which, in turn, increased transformation efficiency (Harwood 2012).

3.2.2.3 Explants and Regeneration Pathways

Somatic embryogenesis and indirect organogenesis are two important morphogenetic alternatives in plant transformation protocols with callus formation steps. The embryogenic callus is both a preferable culture type and suitable target for a monocotyledonous transformation (Anand et al. 2003; Gadaleta et al. 2006; Yue et al. 2008). It was utilised for the transformation of grasses by either particle inflow gun or the biolistic particle delivery system, e.g. *Brachiaria ruziziensis* (Ishigaki et al. 2012), *Pennisetum glaucum* (Goldman et al. 2003), *Bouteloua gracilis* (Santacruz et al. 2009) and *Panicum virgatum* (Xi et al. 2009; Fu et al. 2011). Moreover, efficiently regenerating callus tissue derived from the mature embryos of bread wheat (*Triticum aestivum*) and durum wheat (*T. durum*) cultivars were transformed by particle bombardment (Patnaik and Khurana 2003; Vishnudasan et al. 2005; Ding et al. 2009; Galović et al. 2010; Wang et al. 2012; Li et al. 2012). Although the usage of mature embryos created the problem of low callus induction, which significantly limited their application for genetic engineering, it can be successfully overcome by using

an endosperm-supported callus induction method (Galović et al. 2010). Alternatively, immature embryos can be used with subsequent initiation of morphogenic/embryogenic callus formation for wheat, rye and barley (Pellegrineschi et al. 2002; Popelka and Altpeter 2003; Hensel et al. 2007; Wu et al. 2008; He et al. 2010; Hu et al. 2012; Li et al. 2012; Fujii et al. 2012). Selecting the zygotic embryo developmental stage is one of the most important factors necessary to achieve high responsiveness of callusogenesis and regeneration (Varshney et al. 1996). *Agrobacterium*-mediated co-cultivation of wheat embryogenic calluses derived from immature embryos has yielded 3–9.82 % efficiency in transformation, which can be regarded as high (Vishnudasan et al. 2005; Zhao et al. 2006). In addition to the immature embryos, other wheat explants, such as anther- and inflorescence-derived calluses, apical meristems (Supartana et al. 2006; Zhao et al. 2006) and other floral organs (Agarwal et al. 2009; Zale et al. 2009), were employed in transformation protocols; however, most methods need further optimisation (Li et al. 2012). Young leaves are rarely used in monocots as explants for callus formation. However, the fresh innermost young leaf rolls of mature sugarcane crop plants have been used as a source for the induction of embryogenic calluses and then for the microprojectile-mediated transformation (Ijaz et al. 2012).

In contrast, the immature leaves of dicotyledones are often employed as explants due to rapid callus formation and their high differentiation potential for regeneration (Niaz and Quraishi 2002; Anjum et al. 2012). Strawberry leaf pieces regenerate via organogenesis as dense clusters of emerging shoots after a short callus phase (Hanhineva and Kärenlampi 2007). The leaves are the most important explant source used for the transformation protocols of trees, e.g. sweet cherry, eucalyptus and pear (Sartoretto et al. 2002; Abdollahi et al. 2006; Feeney et al. 2007). The latter protocols involve a step of indirect organogenesis. Besides the leaves in potato, several other plant parts, such as roots, stems and tubers, have been used successfully for callus induction; however, stem segments were found to be the most responsive (Turhan 2004; Heeres et al. 2002; Piqueras et al. 2010). The cotyledons, hypocotyls or petioles of cucumber are sources of embryogenic callus which can be bombarded with microprojectiles coated with plasmid DNAs (Piqueras et al. 2010). The stem internodes and leaf tissue of tobacco (cv. “Samsun NN”) cut transversally into thin cell layers have resulted in green, yellow, white and red callus formed on in vitro and greenhouse explants (Silva 2005).

3.2.2.4 Genotype Versus Transformation Efficiency of Callus

Besides the type of explants, the efficiency of transformation via the callus system strongly differs within the plant genotype. A highly responsive genotype is advantageous because it may be useful in different transformation protocols. One of the most responsive and widely used wheat materials is the progeny of a hybrid between spring wheat lines, known under the generic name of “Bobwhite” (Jones 2005; Jones et al. 2005; Li et al. 2012). It represents a group of 129 accessions in the

Centro Internacional de Melhoramento de Maiz y Trigo (CIMMYT) ex situ collection. The majority of the 129 tested genotypes produced a high yield of somatic embryos, and eight genotypes demonstrated transformation efficiencies above 60 % (60 independent transgenic events per 100 immature embryos bombarded). However, transformation efficiency is not always significantly linked to the embryogenic/morphogenic callus induction capacity. It was demonstrated that the "Jefimija" cultivar, which had one of the highest embryogenic potentials (93 %), exhibited the lowest transformation efficiency. Two other cultivars, with 36 % ("Pesma") and 100 % ("Kantata") induction capacity, had almost identical transformation efficiencies (14.2 and 14.5 %, respectively). These results are in agreement with those of Pellegrineschi et al. (2002), who found that the transformation success is due to the genotypic and physiological status of the donor plants. In barley, the most successful, high-throughput transformation systems use the spring variety "Golden Promise" (Bartlett et al. 2008; Harwood 2012). This genotype has excellent regeneration from immature embryo target tissues, good susceptibility to *Agrobacterium* infection and transformation efficiencies of up to 35 %. High-throughput transformation systems are usually developed for a single responsive genotype and are rarely transferable to alternative genotypes. An ideal transformation system would be genotype independent (Harwood 2012). There has been only one report of a method for the transformation of barley that is considered to be genotype independent. This involves the infection of in vitro-cultured ovules with *Agrobacterium* (Holme et al. 2008). However, ovule isolation in barley, i.e. the target tissue, is a labour-oriented, skilled procedure; therefore, it is not suitable for a high-throughput transformation system.

3.2.2.5 Method of DNA Vector Application

The *Agrobacterium*-mediated gene transfer protocols involving a callus induction step are standard for many dicotyledonous species (Riva et al. 1998; Dandekar and Fisk 2005; Alimohammadi and Bagherieh-Najjar 2009), whereas the restricted host range of *Agrobacterium*-rendered infection of monocots is difficult (Khanna and Daggard 2003; Wu et al. 2009, Ignacimuthu et al. 2000). However, recently *Agrobacterium* transformation efficiency in barley has significantly been raised (Hensel et al. 2007; Bartlett et al. 2008; Fujii et al. 2012). Moreover, Hu et al. (2003) and Travella et al. (2005) showed that *Agrobacterium*-transformed plants had lower transgene copy numbers and more stable transgene expression in barley in comparison to the biolistic method. In wheat, an *Agrobacterium*-mediated transformation is able to yield efficiencies of up to 30 % (Risacher et al. 2009). The immature embryos of hexaploid wheat were used directly as explants for the *Agrobacterium*-mediated transformation with the callus formation in the next step of transgenic plant production (Cheng et al. 1997; Hu et al. 2003; Khanna and Daggard 2003; Wu et al. 2009). Several groups of researchers have inoculated the immature embryos of tetraploid

durum wheat with *Agrobacterium* with an efficiency from 0.6 to 9.7 % depending on the cultivar (Wu et al. 2008; He et al. 2010).

Particle bombardment is the most widely used method for the genetic transformation of most monocots, including wheat (Lazzeri and Jones 2009; Sparks and Jones 2009). Sparks and Jones (2009) describe a biolistic protocol that has allowed the transformation of 35 wheat genotypes, while only a few of those could be transformed using *Agrobacterium*. Maize and rye are commonly transformed by particle bombardment (Frame et al. 2000; Popelka and Altpeter 2003). Particle bombardment can cause serious physical damage to the explant tissues used for transformation, thus negatively affecting the embryogenesis/organogenesis, in vitro regeneration of the explants and, therefore, transformation efficiency.

3.2.3 Cell Suspension Cultures

Cell suspension cultures can be defined as a system of a homogeneous population of individual plant cells suspended in a liquid growth medium. Floating freely, the cells are evenly exposed to all of the nutrients, allow easy manipulation and control of the culture and provide a unique opportunity for studying various metabolic processes (Bhojwani and Razdan 1992; Boisson et al. 2012). Suspensions can be used as a perfect target for genetic transformation due to the large amount of homogeneous material, easy selection of transgenic cells and mitigated risk of chimeric plant regeneration (Finer and McMullen 1991; Cao et al. 1992; González et al. 1998; Ivic and Smigocki 2003, Asaka-Kennedy et al. 2004; Ozawa and Takaiwa 2010).

3.2.3.1 Cell Suspension Initiation and Growth

As has been described in several laboratory manuals (Reinert and Yeoman 1982; Bhojwani and Razdan 1992; Smith 2012), the suspension culture could be started from intact plant organs or in vitro-cultured cells by mechanical or enzymatic cell separation. The mechanical procedures are simple, cheap and less harmful. However, they can be used only with loosely arranged plant tissue, such as leaf mesophyll. The gentle grinding of plant tissue followed by filtration and centrifugation of the received homogenate is used most often. The best plant material for single-cell release is the friable fragments of callus tissue that are transferred to a liquid medium and dispersed by various procedures, e.g. sieving, syringe pumping or enzymatic digestion applied to reduce the aggregate sizes. Once received, the cultures should be agitated vigorously to uniformly distribute the cells in the medium and to stimulate gaseous exchange (Altpeter and Posseh 2000). Proper aeration of the cultures is usually secured by means of an orbital platform shaker with a shaking speed of 30–150 rpm or occasionally by bubbling or stirring (Boisson et al. 2012). In suspension, the growth of the culture follows a specific pattern: the initial short lag phase, exponential growth phase with intensive cell divisions, phase of linear growth, then

progressively decelerated cell divisions and, finally, stationary phase. The duration of each phase mainly depends on the genotypic features and size of the inoculum (Ghosh et al. 2009).

The medium composition should be carefully balanced with respect to many parameters, e.g. nutrients, hormones, pH and osmotic potential (Altpeter and Posseh 2000). The composition of the liquid medium is usually based on the composition of the solid medium that is routinely used for callus culture, although sometimes modifications are necessary. The most common liquid media are MS (Murashige and Skoog 1962), B5 (Gamborg et al. 1968), LS (Linsmaier and Skoog 1965), Blaydes (Blaydes 1966), MX (Nash and Davies 1972), CC (Potrykus et al. 1979) and their further modifications. Generally, the complexity of the medium composition increases along with a decrease in suspension cell density. The very rich synthetic medium as described by Kao and Michayluk (1975) supported cell growth and divisions in a suspension culture at a density of 25 cells per ml. Actively growing suspension cultures utilise a great amount of inorganic phosphate, so liquid media are usually enriched in this compound (Boisson et al. 2012). In some media, additional vitamins and amino and organic acids are used (Ivic and Smigocki 2003; Khanna et al. 2004; Huang et al. 2007; Ghosh et al. 2009; Ozawa and Takaiwa 2010). Similarly to the callus culture, the precise requirement for exogenously applied hormones depends on plant genotype, cell origin and in vitro culture conditions, i.e. the parameters that obviously determine the endogenous level of the growth substances.

Suspension cultures should be subcultured regularly, usually every 1–2 weeks, but the right moment and the dilution ratio depend on the cells' growth and division rates. Frequent subculturing diminishes the threat of carbon substrate consumption, which leads towards the autophagic process and cell death (Roby et al. 1987; Aubert et al. 1996). It usually also minimises the frequency of chromosomal disturbances and sustains the embryogenic potential, which is a prerequisite for its utilisation as a target for genetic transformation (Chen et al. 1998). A long-term suspension culture often loses its morphogenic potential, and after several repeated subcultures, conversion into plants can be markedly decreased or totally lost which could be the result of nuclear instability (polyploidy, aneuploidy or mutations) of in vitro-cultured cells, a disturbed hormonal balance or altered cell sensitivity to growth substances (Bhojwani and Razdan 1992; Dirr and Heuser 2009; Smith 2012). In non-embryogenic suspension cultures of bromegrass, the optimal window for successful transformation was very narrow, i.e. between 7 and 9 days after subculture, which corresponds to a late exponential and early stationary growth stage (Nakamura and Ishikawa 2006). Sometimes, when the loss of morphogenic potential is induced by physiological factors, it can be restored by various treatments, such as low temperature or modification of the medium composition (see in Bhojwani and Razdan 1992). Another approach to solving the problem of a decreasing morphogenic potential is the use of cryopreserved cells stored at ultra-low temperatures, usually −196 °C (Benson 2008). In many cases, re-established cells possessed high competence for plant regeneration, and the plants derived from cryopreserved cells were morphologically and physiologically unchanged (Cornejo et al. 1995; Huang et al. 1995;

Jain et al. 1996; Moukadiri et al. 1999). However, sometimes viability of the unfrozen cells decreased, and long lag phases before full recovery of cell culture growth were necessary (Boisson et al. 2012). Cryopreservation of embryogenic cell suspensions and their use as targets for genetic transformation have been reported in a number of economically important crops (Wang et al. 2012) such as rice (Cho et al. 2007), tobacco (Menges and Murray 2004), cassava (Puonti-Kaerlas et al. 1997), banana (Panis et al. 2005), grapevine (Wang et al. 2005) and sweet potato (Feng et al. 2011). Another approach has recently been reported (Boisson et al. 2012), i.e. suspension cultures of sycamore (*Acer pseudoplatanus*) and *Arabidopsis* were preserved for over 6 months in a phosphate-free nutrient medium at 5 °C to induce cell growth arrest. It took a couple of hours to restart cell growth, and no measurable cell death was observed.

Plant regeneration from cell suspension can follow either organogenesis or somatic embryogenesis pathway, which makes this process similar to plant regeneration from callus culture (Piqueras and Debergh 1999; Kim et al. 2010; Piqueras et al. 2010). However, somatic embryos at the globular stage can be released from the maternal cell clumps in suspension and can further develop into callus tissue clumps by repeated division (see in Bhojwani and Razdan 1992).

3.2.3.2 Cell Suspension as the Target of Transformation

Monocotyledonous crop plants have been the most important targets for genetic transformation via suspension culture. Cell suspensions were initially reported to be a suboptimal explant choice for rice transformation (Hiei et al. 1994) but soon turned out to be an excellent target not only for rice (Urushibara et al. 2001) but also for other species, such as maize, barley, wheat and forage grasses (see Cheng et al. 2004). Until now among others, embryogenic suspension cells have been used as plant material for transgenic plant production in the case of maize (Fromm et al. 1990; Gordon-Kamm et al. 1990), oats (Somers et al. 1992), japonica and indica rice (Cao et al. 1992; Jain et al. 1996; Nandadeva et al. 1999; Urushibara et al. 2001), barley (Ritala et al. 1993; Wu et al. 1998) and forage grasses, e.g. tall and red fescue (Spangenberg et al. 1995a; Dong and Qu 2005; Wang and Ge 2005), perennial and Italian ryegrass (Spangenberg et al. 1995b; Ye et al. 1997; Li et al. 2004; Wu et al. 2005, 2007; Sato and Takamizo 2006; Bajaj et al. 2006), festulolium (*Lolium*/*Festuca* hybrids) (Guo et al. 2009) and orchardgrass (Lee et al. 2006).

The first dicotyledonous species to be transformed with the use of the suspension culture technique were tobacco (Klein et al. 1987, 1988a), carrot (Scott and Draper 1987; Ming-zhi 1996; Hardegger and Sturm 1998), soybean (McCabe et al. 1988; Parrott et al. 1989; Finer and McMullen 1991; Trick and Finer 1998, Rech et al. 2008) and cotton (Finer and McMullen 1990; Rajasekaran et al. 1996, 2000). Then the technique was optimised for cucumber (Schulze et al. 1995; Burza et al. 2006), citrus (Yao et al. 1996; Dutt and Grosser 2010), grapevine (Perl et al. 1995; Wang et al. 2005; Vidal et al. 2006), cassava (Raemakers et al. 1996; Schöpke et al. 1996; González et al. 1998; Schreuder et al. 2001), sugarcane (Arencibia et al. 1998) and

melon (Guis et al. 2000). Recently, efficient protocols have also been established for sugar beet (Ivic and Smigocki 2003); banana (Khanna et al. 2004; Huang et al. 2007; Ghosh et al. 2009; Ganapathi et al. 2001); some forage legumes like alfalfa, white clover and *Medicago truncatula* (Kalla et al. 2001; Zhang et al. 2005; Balance and McManus 2006; Crane et al. 2006; Wright et al. 2006; Xie et al. 2006; Montague et al. 2007; Rosellini et al. 2007; Barone et al. 2008) and sweet potato (Zang et al. 2009; Yang et al. 2011).

3.2.3.3 Suspension Culture Versus Various Transformation Systems

Agrobacterium tumefaciens was rarely used for transformation of the suspension cells of monocotyledonous plants (Newell 2000). Wu et al. (1998) first described the *Agrobacterium tumefaciens*-mediated transformation in a barley suspension culture initiated from immature embryo-derived and microspore-derived callus. This system was also used for an efficient wheat suspension transformation (Weir et al. 2001). A few more reports describe the successful transformation of japonica (Lucca et al. 2001) and indica rice (Hoa and Bong 2003) with the use of embryogenic suspension cultures derived from friable callus tissue that is initiated from mesocotyl tissue, which then results in a transformed cell line production. In 2006, Nakamura and Ishikawa reported the transformation of non-embryogenic bromegrass (*Bromus inermis*) suspension.

Among the dicotyledonous plants the *Agrobacterium* method is often used, and effective production of transgenic plants has been received in most cases for embryogenic cell suspension cultures initiated from friable callus tissue. A carrot suspension culture initiated from callus tissue was used for this purpose in several studies (Scott and Draper 1987; Ming-zhi 1996; Hardegger and Sturm 1998). The genetic transformation of sweet potato (*Ipomoea batatas*) was conducted utilising a suspension culture initiated from shoot bud-derived callus (Yang et al. 2011). The centrifugation-assisted *Agrobacterium*-mediated transformation of banana cultivars (Musa ssp.) was efficient for all three economically important genomic groups (AAA, AAB and AA) (Khanna et al. 2004; Huang et al. 2007). The embryogenic cell suspension of Musa ssp. can be established using an immature male flower culture (Ghosh et al. 2009). In the case of several citrus species, embryogenic cell suspension cultures were initiated from unfertilised ovules (Dutt and Grosser 2010). Embryogenic cell suspensions are also the most often used target tissues for transformation studies in grapevine (Perl et al. 1995; Wang et al. 2005). In this species, improvement of *Agrobacterium*-mediated transformation efficiency and transgenic plant regeneration was achieved by the use of cryopreserved cell suspensions initiated from anther-derived callus tissue (Wang et al. 2005).

Compared with forage grasses, transformation is relatively easier in forage legume species. Efficient protocols for the genetic transformation of alfalfa, white clover and *Medicago truncatula* using *A. tumefaciens* have been established over the last decade (Kalla et al. 2001; Zhang et al. 2005; Balance and McManus 2006; Crane et al. 2006; Wright et al. 2006; Xie et al. 2006; Montague et al. 2007; Rosellini et al. 2007; Barone et al. 2008).

Cell suspension cultures serve as ideal target material for a biolistic transformation (Christou 1992), as in a relatively short period of time it can generate a large number of homogeneous cells (Ivic and Smigocki 2003) and has been used successfully to transform several monocotyledonous and dicotyledonous species (Kamo et al. 1995). The technique was first successfully applied to the suspension cells of *Nicotiana tabacum* (Klein et al. 1987, 1988a) and then *Zea mays* (Klein et al. 1988b). A system for the generation of fertile, transgenic plants by the microprojectile bombardment of embryogenic suspension cultures from immature embryos was developed for maize by Gordon-Kamm et al. (1990). Suspension cultures produced from the scutella of immature embryos were also used for rice transformation (Chen et al. 1998; Nandadeva et al. 1999) and Russian wildrye (*Psathyrostachys juncea*) plants, a forage species well adapted to semi-arid climates (Wang et al. 2004). Furthermore, microprojectile bombardment has been used to transform embryogenic suspension cultures from the friable callus of soybean (McCabe et al. 1988; Parrott et al. 1989; Finer and McMullen 1991; Rech et al. 2008). The biolistic method, with cell suspension initiated from leaf-derived callus tissues, was used for transgenic cucumber (*Cucumis sativus* L.) (Schulze et al. 1995) and sugar beet (Ivic and Smigocki 2003). Moreover, this transformation method can be applied to cryopreserved cultures (Rajasekaran et al. 1996). A stable transformation of cotton (*Gossypium hirsutum* L.) has been obtained by multiple bombardments of cryopreserved cultures of embryogenic cell suspension cultures during the rapid growth phase (Finer and McMullen 1990; Rajasekaran et al. 1996, 2000).

A method strictly applied to suspension cultures is the one described by Wang et al. (1995) with the use of *silicon carbide* (SiC) *whiskers* for cell penetration. The technique involves the mixing of plant cells and plasmid DNA with SiC whiskers, which have great hardness and possess sharp cutting edges. This facilitates DNA delivery by cell perforation and abrasion during the mixing process. However, the technique, although inexpensive and simple, is not widely applied, as only suspensions of high regeneration potential can be used as the transformation object. The method was first used for the cell suspension of maize (*Zea mays*) and tobacco (*Nicotiana tabacum*) by Kaeppler et al. (1990) and Frame et al. (1994). Moreover, transgenic perennial ryegrass plants were produced with the use of embryogenic cell suspension cultures by (SiC) fibre methods (Dalton et al. 1998), although effectiveness of the process was very low.

3.2.3.4 Microspore Suspension Cultures

Microspore suspension culture can be regarded as a separate variant of cell suspension culture. Microspores are haploid cells resulting from a meiotic division in the male reproductive organs of a plant, i.e. the anthers. They are originally designed to develop into pollen (Wędzony et al. 2009; Dubas et al. 2012). In microspore suspension cultures, pollen formation is artificially inhibited by the application of various stress treatments (Shariatpanahi et al. 2006), and the microspores are induced to form multicellular, usually haploid, embryo-like structures called

"gametic embryos" (Dubas et al. 2010, 2011) or cell clumps of irregular shape (Barinova et al. 2004; Wędzony et al. 2009). Both types of structures can regenerate complete plants under appropriate conditions. After duplication of the chromosome number at either of the developmental stages, from microspore to plant, a completely homozygotic line is obtained, called a doubled haploid (DH) line. Microspore cultures offer a multiple and synchronous population of haploid cells (Dunwell 1996; Touraev et al. 1996a, b; Ferrie and Caswell 2011), which are valued in combination with different transformation protocols. The type of microspore isolation method used significantly influences the efficiency of androgenic induction and the number of plants obtained. Microspores can be obtained upon homogenisation of whole spikes or isolated anthers in a micro-blender (Jähne and Lörz 1995; Kasha et al. 2001; Zur et al. 2008, 2009). Indirectly, microspores can be released from an anther floating culture (shed-microspore culture), which precedes microspore re-culture or isolation by pestle maceration (Jähne and Lörz 1995; Dubas et al. 2010, 2011). A great number of factors determine the utility and efficiency of the microspore suspension method: genotype, developmental stage of the microspores and type of stress applied to androgenesis induction (reviewed by Wędzony et al. 2009).

Cold is the most common stress signal (reviewed by Wędzony et al. 2009); however, heat treatments applied to the whole plant, donor inflorescence or isolated anthers prior to culture are also applied to induce androgenesis in the microspores (Coumans et al. 1989; Mejza et al. 1993). Starvation stress is achieved by the culture in mannitol medium (Hoekstra et al. 1996; Touraev et al. 1996a, b; Kasha et al. 2001; Wędzony et al. 2009; Zur et al. 2008, 2009; Dubas et al. 2010), while osmotic stress can be enhanced by polyethylene glycol added to the culture medium (Ilic-Grubor et al. 1998; Delaitre et al. 2001; Lionneton et al. 2001; Ferrie and Keller 2004).

MS (Murashige and Skoog 1962), B5 (Gamborg et al. 1968), H (Nitsch and Nitsch 1969) and AT3 (Touraev and Heberle-Bors 1999) are the most popular as a basic medium. Maltose or sucrose is the most commonly applied sources of organic carbon, thus playing the role of the osmoticum at the same time (Indrianto et al. 1999; Mejza et al. 1993; reviewed by Wędzony et al. 2009). For crop microspore culture, hormone-free media are often used; however, there are strong interactions of the protocols with the genotypes. When applied, the most common auxins or auxin analogues are IAA, PAA, 2,4-D, picloram and dicamba (Wędzony et al. 2009), often in combination with kinetin, zeatin, BA or BAP (Castillo et al. 2000; reviewed by Wędzony et al. 2009). To enhance the regeneration rate, abscisic acid (Hoekstra et al. 1996; Wang et al. 1999) and extracellular arabinogalactans (AGPs) (Kasha et al. 2001; Borderies et al. 2004) have been used. Activated charcoal and colchicine were also found to raise the overall efficiency of microspore androgenesis (Zhao et al. 1996a, b; Zheng et al. 2001; Zhou et al. 2002a, b). As a substitute for exogenous growth regulators, ovaries are applied as a source of auxins and other unidentified compounds (Zheng et al. 2001; Liu et al. 2002a, b; Wędzony et al. 2009).

Starting with the fourth week of culture, well-developed embryo-like structures can be transferred to regeneration conditions. The regeneration phase usually proceeds on solid media: half-strength MS (pH 5.8; Murashige and Skoog 1962) or B5

(Gamborg et al. 1968) medium supplemented with different concentrations of growth regulators (BA, NAA, kinetin, IBA for shoot and GA_3 for rooting). The addition of abscisic acid improves regeneration in cereals. The regenerated plants are finally treated with colchicine to duplicate the chromosome numbers in their cells. However, in some genotypes this step can be omitted due to the sufficient spontaneous duplication of the chromosome number during culture. For example, a spontaneous chromosome doubling rate among microspore-derived wheat plants is only 15–25 %, while for barley 70–80 % has been reported (Kahrizi and Mohammadi 2009; Kahrizi 2009).

The combination of in vitro androgenesis and genetic transformation has been successfully applied to many species for the rapid development of fully homozygous transgenic lines. Transgenic, microspore-derived plants include barley, maize, rice and wheat (review in Jähne and Lörz 1995) and other economically important species such as tobacco (Stöger et al. 1995), oilseed rape (Nehlin et al. 2000, review in Ferrie and Möllers 2010) and sunflower (Weber et al. 2003). Several methods of transformation have been applied to transfer genes to the microspore containing the haploid plant genome. The transformation methods are basically the same as described above for the cell suspension cultures (Jardinaud et al. 1993, review in Touraev et al. 2009). Particle bombardment was described as a suitable method for tobacco (Stöger et al. 1995), oilseed rape (Fukuoka et al. 1998), barley (review in Jähne and Lörz 1995; Yao et al. 1997; Carlson et al. 2001), wheat (Mentewab et al. 1999; Folling and Olesen 2001), maize (Wright et al. 2001) and conifers (reviewed by Luthra et al. 1997). In many species the *Agrobacterium*-mediated methods are favoured because of their ease of implementation and low cost (reviewed by Maheshwari et al. 2011). They have been successfully used for late uni- to early bi-nucleated microspores or the callus derived from them in barley (review in Jähne and Lörz 1995; Nehlin et al. 2000).

3.2.4 Protoplasts

Protoplast culture is a kind of suspension culture and represents the finest single-cell system which is reliable as long as their source is selected carefully (Faraco et al. 2011). Protoplasts of higher plants can introduce foreign DNA, cell organelles, bacteria or virus particles through their exposed plasma membrane. These unique properties, combined with the totipotent nature of plant cells, make protoplasts excellent starting material for generating unique and novel plants (Mendes et al. 2001; Ishikawa et al. 2003).

The totipotency and durability of protoplasts are some of the most important criteria for protoplast fusion or genetic transformation. Protoplasts can be isolated from in vitro tissue cultures (mainly callus or suspension cell cultures). Also, tissue organs and specialised groups of cells, such as roots, shoots, leaves, fragments of flowers or pollen grains, can be used to obtain protoplasts (Sheen 2001; Grzebelus et al. 2012).

The removal of the cell wall can proceed in two ways: by mechanical and enzymatic digestion. Mechanical procedures, involving the slicing of plasmolysed tissues, are currently rarely employed for protoplast isolation, but they are useful with large cells and when limited (small) numbers of protoplasts are required. Normally, when large populations of protoplasts are required the enzymatic digestion of source tissues is essential (Eriksson 1985; Davey et al. 2000; Aoyagi 2006). Several factors influence protoplast release, including the extent of the thickening of cell walls, temperature, duration of enzyme incubation, pH optima of the enzyme solution (Sinha et al. 2003), gentle agitation, and nature of the osmoticum. Freshly isolated protoplasts are spherical because they are unrestricted by the cell wall. When given the correct chemical and physical stimuli, each protoplast is capable of regenerating a new wall and undergoing repeated mitotic division to produce daughter cells, from which fertile plants may be regenerated via the tissue culture process. The culture of protoplasts is conducted on liquid, semi-solid or solid media. Many media have been based on the MS (Murashige and Skoog 1962) and B5 (Gamborg et al. 1968) formulations, with the addition of an osmoticum (Sato et al. 1993; Winkelmann et al. 2006; Badr-Elden et al. 2010).

Protoplasts are used as starting materials to transfer foreign genes into plants in a process called protoplast fusion. The general method of protoplast fusion is, first, combining the parental protoplasts to obtain a mixture of protoplasts derived from different parental cell types. There are two ways to fuse protoplasts: (1) chemically induced fusion and (2) electrofusion. The thrust of the first method is to overcome the net of negative surface charge on the protoplasts by treating them with chemical fusogens. The most common chemical used to stimulate DNA is PEG, i.e. polyethyleneglycol, which increases the permeability of cell membranes (Potrykus et al. 1985; Nugent et al. 2006). The disadvantage of using this method is its unpredictability, because it cannot be used on some plant species and also because the chemical fusion frequency may vary for a given species. Electrofusion is a method of using short electrical pulses of high field strength to facilitate DNA uptake by increasing the permeability of the protoplast membranes. Usually, a high-frequency alternating field of 0.5–1.5 MHz is applied across two electrodes to stimulate protoplast migration. The second step of electrofusion is to apply one or more short direct current pulses to cause reversible membrane breakdown. This method is used in many crops (Matsumoto et al. 2002; Zheng et al. 2003; Ge et al. 2006; Sharma et al. 2011). The fusion frequency is affected by a number of factors, e.g. different populations of protoplasts will vary in the type of the output product. The large-sized protoplasts fuse more readily than the smaller ones. A longer length of chains of protoplasts will lead to a higher fusion frequency, there will be more multifusion products if a longer pulse at a higher voltage is used and the inclusion of ions in the fusion medium increases the percentage of the fusion. However, it is notable that too long pulses at too high a voltage can kill the protoplasts.

Another method of protoplast transformation is electroporation (Bates 1999). This is a technique that uses an electrical pulse to render cell walls or protoplast membranes permeable so that DNA can be taken up into the cells. A high-voltage electrical pulse of shorter duration causes the formation of temporary pores which

allows cells to take up plasmid DNA; this may lead to stable or transient DNA expression. The method was originally applied to protoplasts (Zhang et al. 1988; He et al. 1994) but has been found applicable to cells and even tissues (D'Halluin et al. 1992). Joersbo and Brunstedt (1990) introduced DNA into sugar beet and tobacco protoplasts by applying a brief exposure of 20 kHz ultrasound in the presence of a plasmid containing the CAT gene fused to the 35S promoter. The method is known as the "sonication method", and its advantage is that the system may be simpler than that of electroporation.

Microinjection is one of the most precise techniques for delivering foreign DNA into specific compartments of protoplasts (Crossway et al. 1986), but it is not routinely used (Jones-Villeneuve et al. 1995). Microinjections allow to introduce not only plasmids but also whole chromosomes into the plant cells (Griesbach 1987). Several plant species, such as tobacco (Schnorf et al. 1991), oilseed rape (Neuhaus et al. 1987) and barley (Holm et al. 2000), have been transformed using this technique.

3.3 Conclusions

This chapter shows the great variability of possible in vitro culture technologies that are suitable for plant transformation. A range of protocols is available for some cultivated crops and for the model plant, *Arabidopsis thaliana*. Protocols for rare ornamental or medicinal plants are fewer in number. When preparing experiments with a new genotype, we need to have in mind that all methods are genotype dependent; therefore, even a protocol elaborated for the same species might not give the expected results across all genotypes and it will need fine tuning and adjustments to the conditions available in the laboratory and to the genotype under investigation.

The basic research that tries to explain molecular mechanisms beyond the in vitro processes is making progress, but it also raises new questions at the same time. We are far from understanding all of the regulation pathways affecting in vitro callusogenesis, organogenesis or somatic embryogenesis. Thus, most of the protocols have been developed by experimenting with many options, followed by the selection of the most effective variants. The efficiency of in vitro protocols is the result of numerous processes and it is under complex genetic regulation; thus, dissecting all of them would be equal to understanding the mystery of life. Science is persistently on its way to achieving that goal.

References

Abdollahi H, Muleo R, Rugini E (2006) Optimisation of regeneration and maintenance of morphogenic callus in pear (*Pyrus communis* L.) by simple and double regeneration techniques. Sci Hortic 108:352–358

Aboshama HMS (2011) Direct somatic embryogenesis of pepper (*Capsicum annuum* L.). World JAgric Sci 7:755–762

Agarwal S, Loar S, Steber CM, Zale J (2009) Floral transformation of wheat. Methods Mol Biol 478:105–114

Alam I, Sharmin SA, Mondal SC, Alam MJ, Khalekuzzaman M, Anisuzzaman M, Alam MF (2010) *In vitro* micropropagation through cotyledonary node culture of castor bean (*Ricinus communis* L.). Aust J Crop Sci 4:81–84

Alimohammadi M, Bagherieh-Najjar MB (2009) *Agrobacterium*-mediated transformation of plants: basic principles and influencing factors. Afr J Biotechnol 8:5142–5148

Altpeter F, Posseh UK (2000) Improved plant regeneration from cell suspensions of commercial cultivars, breeding and inbred lines of perennial ryegrass (*Lolium perenne* L.). J Plant Physiol 156:790–796

Anand A, Zhou T, Trick HN, Gill BS, Bockus WW, Muthukrishnan S (2003) Greenhouse and field testing of transgenic wheat plants stably expressing genes for thaumatin-like protein, chitinase and glucanase against *Fusarium graminearum*. J Exp Bot 54:1101–1111

Anjum N, Ijaz S, Rana IA, Khan TM, Khan IA, Khan MN, Mustafa G, Joyia FA, Iqbal A (2012) Establishment of an in vitro regeneration system as a milestone for genetic transformation of sugarcane (*Saccharum officinarum* L) against Ustilago scitaminea. Biosci Methods 3:7–20

Anwar F, Sharmila P, Saradhi PP (2010) No more recalcitrant: Chickpea regeneration and genetic transformation. Afr J Biotechnol 9:782–797

Aoyagi H (2006) Development of a quantitative method for determination of the optimal conditions for protoplast isolation from cultured plant cells. Biotechnol Lett 28:1687–1694

Arencibia AD, Carmona ERC, Tellez P, Chan M-T, Yu S-M, Trujillo LE, Oramas P (1998) An efficient protocol for sugarcane (*Saccharum* spp. L.) transformation mediated by *Agrobacterium tumefaciens*. Transgenic Res 7:213–222

Asaka-Kennedy Y, Tomita K, Ezura H (2004) Efficient plant regeneration and *Agrobacterium-mediated* transformation via somatic embryogenesis in melon (*Cucumis melo* L.). Plant Sci 166:763–769

Aubert S, Gout E, Bligny R, Mazars-Marty D, Barrieu F, Alabouvette J, Marty F, Douce R (1996) Ultrastructural and biochemical characterization of autophagy in higher plant cells submitted to carbon deprivation: control by the supply of mitochondria with respiratory substrates. J Cell Biol 133:1251–1263

Aviles-Vinas SA, Lecona-Guzman CA, Canto-Flick A, Lopez-Erosa S, Santana-Buzzy N (2012) Morpho-histological and ultrastructural study on direct somatic embryogenesis of *Capsicum Chinese* Jacq. in liquid medium. Plant Biotechnol Rep. doi10.1007/s11816-012-0261-0

Badr-Elden AM, Nower AA, Nasr MI, Ibrahim AI (2010) Isolation and fusion of protoplasts in sugar beet (*Beta vulgaris* L.). Sugar Technol 12:53–58

Bajaj S, Ran Y, Phillips J, Kulrajathevan G, Pal S, Cohen D, Elborough K, Sathish P (2006) A high throughput *Agrobacterium* tumefaciens-mediated transformation method for functional genomics of perennial ryegrass (*Lolium perenne* L.). Plant Cell Rep 25:651–659

Balance C-MC, McManus MT (2006) Expression of 1-aminocyclopropane-1-carboxylate (ACC) oxidase genes during the development of vegetative tissues in white clover (*Trifolium repens* L.) is regulated by ontological cues. Plant Mol Biol 60:451–467

Banerjee P, Sharmistha MS, Banerjee N (2012) High frequency somatic embryogenesis and plantlet regeneration of *Bauhinia variegata*, a multipurpose tree legume. Indian J Fundam Appl Life Sci 2:87–95

Barinova I, Clement C, Martiny L, Baillieul F, Soukupova H, Heberle-Bors E, Touraev A (2004) Regulation of developmental pathways in cultured microspores of tobacco and snapdragon by medium pH. Planta 219:141–146

Barone P, Rosellini D, LaFayette P, Bouton J, Veronesi F, Parrot W (2008) Bacterial citrate synthase expression and soil aluminum tolerance in transgenic alfalfa. Plant Cell Rep 27: 893–901

Barro F, Martin A, Lazzeri PA, Barcelo P (1999) Medium optimization for efficient somatic embryogenesis and plant regeneration from immature inflorescences and immature scutella of elite cultivars of wheat, barley and tritordeum. Euphytica 180:161–167

Bartlett JG, Alves SC, Smedley M, Snape JW, Harwood WA (2008) High-throughput *Agrobacterium*-mediated barley transformation. Plant Methods 4:1–12

Bates GW (1999) Plant transformation via protoplast electroporation. Methods Mol Biol 111: 359–366

Bele D, Tripathi MK, Tiwari G, Baghel BS, Tiwari S (2012) Microcloning of sandalwood (*Santalum album* Linn.) from cultured leaf discs. J Agric Technol 8:571–583

Benson EE (2008) Cryopreservation of phytodiversity: a critical appraisal of theory and practice. Crit Rev Plant Sci 27:141–219

Bhagyawant SS, Behra KK, Mishra S, Sharma A, Srivastava N (2012) Influence of light stress on somatic embryos inducing invitro antimicrobial activity in *Carthamus tinctorius* L. (variety-Mangira). J. J Pharm Res 5:2505–2509

Bhojwani SS, Razdan MK (1992) Plant tissue culture: theory and practice. Elsevier, Amsterdam, p 776

Blaydes DF (1966) Interaction of kinetin and various inhibitors in the growth of soybean tissues. Physiol Plant 19:748–753

Boisson A-M, Gout E, Bligny R, Rivasseau C (2012) A simple and efficient method for the long-term preservation of plant cell suspension cultures. Plant Methods 8:4–16

Borderies G, le Béchec M, Rossignol M, Laflitte C, Deunnff E, Beckert M, Dumas C, Matthys-Rochon E (2004) Characterization of proteins secreted during maize microspore culture: arabinogalactan proteins (AGPs) stimulate embryo development. Eur J Cell Biol 83:205–212

Borna RS, Hoque MI, Sarker RH (2010) *Agrobacterium*-mediated genetic transformation for local cultivars of potato (*Solanum tuberosum* L.) using marker genes. Plant Tiss Cult Biotechnol 20:145–155

Bregitzer P, Campbell R (2001) Genetic markers associated with green and albino plant regeneration from embryogenic barley callus. Crop Sci 41:173–179

Burza W, Zuzga S, Yin ZM, Malepszy S (2006) Cucumber (*Cucumis sativus* L.). In: Wang K (ed) Agrobacterium protocols, vol 2, 2nd edn. Humana, Totowa, NJ, pp 427–438

Cai Z, Kastell A, Knorr D, Smetanska I (2012) Exudation: an expanding technique for continuous production and release of secondary metabolites from plant cell suspension and hairy root cultures. Plant Cell Rep 31:461–477

Cao J, Duan X, McElroy D, Wu R (1992) Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. Plant Cell Rep 11:586–591

Carlson AR, Letarte J, Chen J, Kasha KJ (2001) Visual screening of microspore-derived transgenic barley (*Hordeum vulgare* L.) with green-fluorescent protein. Plant Cell Rep 20:331–337

Castillo AM, Vallés MP, Cistué L (2000) Improvements in barley androgenesis for plant breeding. In: Bohanec B (ed) Biotechnological approaches for utilization of gametic cells. COST 824, Bled, Slovenia, pp 15–21

Chen L, Zhang S, Beachy RN, Fauquet CM (1998) A protocol for consistent, large-scale production of fertile transgenic rice plants. Plant Cell Rep 18:25–31

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, Wan Y (1997) Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiol 115:971–980

Cheng M, Lowe BA, Spencer TM, Ye X, Armstrong CL (2004) Factors influencing *Agrobacterium-mediated* transformation of monocotyledonous species. In Vitro Cell Dev Biol Plant 40: 31–45

Cho JS, Hong SM, Joo SY, Yoo JS, Kim DI (2007) Cryopreservation of transgenic rice suspension cells producing recombinant hCTLA4Ig. Appl Microbiol Biotechnol 73:1470–1476

Christou P (1992) Genetic transformation of crop plants using microprojectile bombardment. Plant J 2:275–281

Cornejo MJ, Wong VL, Blechl AE (1995) Cryopreserved callus: a source of protoplasts for rice transformation. Plant Cell Rep 14:210–214

Coumans MP, Sohota S, Swanson EB (1989) Plant development from isolated microspores of *Zea mays* L. Plant Cell Rep 7:618–621

Crane C, Wright E, Dixon RA, Wang Z-Y (2006) Transgenic *Medicago truncatula* plants obtained from *Agrobacterium* tumefaciens-transformed roots and *Agrobacterium* rhizogenes-transformed hairy roots. Planta 223:1344–1354

Crossway A, Oakes JV, Irvine JM, Ward B, Knauf VC, Shewmaker CK (1986) Integration of foreign DNA following microinjection of tobacco mesophyll protoplasts. Mol Gen Genet 202:179–185

da Silva JAT (2005) Simple multiplication and effective genetic transformation (four methods) of in vitro-grown tobacco by stem thin cell layers. Plant Sci 169:1046–1058

Dalton SJ, Bettany AE, Timms E, Morris P (1998) Transgenic plants of *Lolium multiflorum*, *Lolium perenne*, *Festuca arundinacea* and *Agrostis stolonifera* by silicon carbide fibre-mediated transformation of cell suspension cultures. Plant Sci 132:31–43

Dan Y, Armstrong CL, Dong J, Feng X, Fry JE, Keithly GE, Martinell BJ, Roberts GA, Smith LA, Tan LJ, Duncan DR (2009) Lipoic acid: a unique plant transformation enhancer. In Vitro Cell Dev Biol Plant 45:630–638

Dandekar AM, Fisk HJ (2005) Plant transformation: *Agrobacterium*-mediated gene transfer. Methods Mol Biol 286:35–46

Davey MR, Power JB, Lowe KC (2000) Plant protoplats. In: Spier RE (ed) Encyclopaedia of cell technology. Wiley, New York, pp 1034–1042

de la Riva GA, Cabrera JG, Padrón RV, Pardo CA (1998) *Agrobacterium tumefaciens*: a natural tool for plant transformation. EJB Eur J Biotechnol 3:1–16

Delaitre C, Ochatt SJ, Deleury E (2001) Electroporation modulates the embryogenic responses of asparagus (*Asparagus officinalis* L.) microsopres. Protoplasma 216:39–46

Dey M, Bakshi S, Galiba G, Sahoo L, Panda SK (2012) Development of a genotype independent and transformation amenable regeneration system from shoot apex in rice (*Oryza sativa* spp. *indica*) using TDZ. Biotechnology 2:233–240

D'Halluin K, Bonne E, Bossut M, De Beuckeleer M, Leemans J (1992) Transgenic maize plants by tissue electroporation. Plant Cell 4:1495–1505

Ding L, Li S, Gao J, Wang Y, Yang G, He G (2009) Optimization of *Agrobacterium*-mediated transformation conditions in mature embryos of elite wheat. Mol Biol Rep 36:29–36

Dirr MA, Heuser JCW (2009) The reference manual of woody plant propagation: from seed to tissue culture, 2nd ed. pp. 424. Timber, USA

Dong S, Qu R (2005) High efficiency transformation of tall fescue with *Agrobacterium* tumefaciens. Plant Sci 168:1453–1458

Dubas E, Custers J, Kieft H, Wędzony M, van Lammeren AAM (2011) Microtubule configurations and nuclear DNA synthesis during initiation of suspensor-bearing embryos from *Brassica napus* cv. Topas microspores. Plant Cell Rep 30:2105–1216

Dubas E, Wędzony M, Petrovska B, Salaj J, Zur I (2010) Cell structural reorganization during induction of androgenesis in isolated microspore cultures of triticale (x *Triticosecale* Wittm.). Acta Biol Crac Ser Bot 52:73–86

Dubas E, Wędzony M, Custers J, Kieft H, van Lammeren AAM (2012) Gametophytic development of *Brassica napus* pollen *in vitro* enables examination of cytoskeleton and nuclear movements. Protoplasma 249:369–377

Dunwell JM (1996) Microspore culture. In: Jain SM, Sopory SK, Veilleux RE (eds) *In vitro* haploid production in higher plants: fundamental aspects and methods. Kluwer Academic Publishers, Dordrecht, pp 205–216

Dutt M, Grosser JW (2010) An embryogenic suspension cell culture system for *Agrobacterium-mediated* transformation of citrus. Plant Cell Rep 29:1251–1260

Eriksson TR (1985) Protoplast isolation and culture. In: Fowke LC, Constabel F (eds) Plant protoplasts. CRC, Boca Raton, FL, pp 1–20

Faraco M, Sansebastiano PD, Spelt K, Koes RE, Quattrocchio FM (2011) One protoplast is not the other! Plant Physiol 156:474–478

Feeney M, Bhagwat B, Mitchell JS, Lane WD (2007) Shoot regeneration from organogenic callus of sweet cherry (*Prunus avium* L.). Plant Cell Tiss Cult 90:201–214

Feng C, Yin Z, Ma Y, Zhang Z, Chen L, Wang B, Li B, Huang Y, Wang Q (2011) Cryopreservation of sweetpotato (*Ipomoea batatas*) and its pathogen eradication by cryotherapy. Biotechnol Adv 29:84–93

Ferrie AMR, Mollers C (2010) Haploids and doubled haploids in Brassica spp. for genetic and genomic research. Plant Cell Tiss Org Cult 104:375–386

Ferrie AMR, Caswell KL (2011) Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. Plant Cell Tiss Org Cult 104:301–309

Ferrie AMR, Keller WA (2004) Brassica improvement through microspore culture. In: Pua EC, Douglas CJ (eds) Biotechnology in agriculture and forestry 54: Brassica. Springer, Berlin, pp 149–168

Finer JJ, McMullen MD (1990) Transformation of cotton (*Gossypium hirsutum* L.) via particle bombardment. Plant Cell Rep 8:586–589

Finer JJ, McMullen MD (1991) Transformation of soybean via particle bombardment of embryogenic suspension culture tissue. In Vitro Cell Dev Biol Plant 27:175–182

Folling L, Olesen A (2001) Transformation of wheat (*Triticum aestivum* L.) microspore-derived callus and microspores by particle bombardment. Plant Cell Rep 20:629–636

Frame BR, Drayton PR, Bagnall SV, Lewnau CJ, Bullock WP, Wilson HM, Dunwell JM, Thompso JA, Wang K (1994) Production of fertile transgenic maize plants by silicon carbide whisker-mediated transformation. Plant J 6:941–948

Frame BR, Zhang H, Cocciolone SM, Sidorenko LV, Dietrich CR, Pegg SE, Zhen S, Schnable PS, Wang K (2000) Production of transgenic maize from bombarded Type II callus: effect of gold particle size and callus morphology on transformation efficiency. In Vitro Cell Dev Biol Plant 36:21–29

Fromm ME, Morrish A, Armstrong R, Williams J, Thomas J, Klein TM (1990) Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Biotechnology 8:833–83

Fu C, Xiao X, Xi Y, Ge Y, Chen F, Bouton J, Dixon RA, Wang ZY (2011) Downregulation of cinnamyl alcohol dehydrogenase (CAD) leads to improved saccharification efficiency in switchgrass. Bioenerg Res 4:153–164

Fujii M, Yokosho K, Yamaji N, Saisho D, Yamane M, Takahashi H, Sato K, Nakazono M, Ma JF (2012) Acquisition of aluminium tolerance by modification of a single gene in barley. Nat Commun 3:713

Fukuoka H, Ogawa T, Matsuoka M, Ohkawa Y, Yano H (1998) Direct gene delivery into isolated microspores of rapeseed (*Brassica napus* L.) and the production of fertile transgenic plants. Plant Cell Rep 17:323–328

Gadaleta A, Giancaspro A, Blechl A, Blanco A (2006) Phosphomannose isomerase, pmi, as a selectable marker gene for durum wheat transformation. J Cereal Sci 43:31–37

Galović V, Rausch T, Gršić-Rausch S (2010) Mature embryo-derived wheat transformation with major stress modulated antioxidant target gene. Arch Biol Sci Belgrade 62:539–546

Gamborg OL, Miller RA, Ojima O (1968) Nutrient requirements of suspension cultures of soybean root cell. Exp Cell Res 50:151–158

Ganapathi TR, Higgs NS, Balint-Kurti PJ, Van Eck J (2001) *Agrobacterium*-mediated transformation of embryogenic cell suspensions of banana cultivar Rasthali (AAB). Plant Cell Rep 20:157–162

Ganeshan S, Baga M, Harvey BL, Rossnagel BG, Scoles GJ, Chibbar RN (2003) Production of multiple shoots from thidiazuron-treated mature embryos and leaf-base/apical meristems of barley (*Hordeum vulgare*). Plant Cell Tiss Org Cult 73:57–64

Ge TM, Lin XH, Qin NFL, Yu SW, Yu YJ (2006) Protoplast electrofusion between common wheat (*Triticum aestivum* L.) and Italian ryegrass (*Lolium multiflorum* Lam.) and regeneration of mature cybrids. In Vitro Cell Dev Biol Plant 42:179–187

Ghosh A, Ganapathi TR, Nath P, Bapat VA (2009) Establishment of embryogenic cell suspension cultures and *Agrobacterium*-mediated transformation in an important *Cavendish banana* cv. Robusta (AAA). Plant Cell Tiss Org Cult 97:131–139

Goldman JJ, Hanna WW, Fleming G, Ozias-Akins P (2003) Fertile transgenic pearl millet (*Pennisetum glaucum* L.) plants recovered through microprojectile bombardment and

phosphinothricin selection of apical meristem-, inflorescence-, and immature embryo-derived embryogenic tissues. Plant Cell Rep 21:999–1009

González AE, Schöpke C, Taylor NJ, Beachy RN, Fauquet CM (1998) Regeneration of transgenic cassava plants (*Manihot esculenta* Crantz) through *Agrobacterium*-mediated transformation of embryogenic suspension cultures. Plant Cell Rep 17:827–831

Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams WR Jr, Willetts NG, Rice TB, Mackey CJ, Krueger RW, Kausch AP, Lemaux PG (1990) Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2:603–618

Greer MS, Kovalchuk I, Eudes F (2009) Ammonium nitrate improves direct somatic embryogenesis and biolistic transformation of *Triticum aestivum*. New Biotechnol 26:44–52

Griesbach RJ (1987) Chromosome mediated transformation via microinjection. Plant Sci 50:69–77

Grzebelus E, Szklarczyk M, Baranski R (2012) An improved protocol for plant regeneration from leaf and hypocotyl-derived protoplasts of carrot. Plant Cell Tiss Org Cult 109:101–109

Guis M, Ben Amor M, Latché A, Pech JC, Roustan JCA (2000) Reliable system for the transformation of cantaloupe Charentais melon (*Cucumis melo* L. var. *cantalupensis*) leading to a majority of diploid regenerants. Sci Hortic 84:91–99

Guo Y-D, Hisano H, Shimamoto Y, Yamada T (2009) Transformation of androgenic-derived Festulolium plants (*Lolium perenne* L. x *Festuca pratensis* Huds.) by *Agrobacterium* tumefaciens. Plant Cell Tiss Org Cult 96:219–227

Hanhineva KJ, Kärenlampi SO (2007) Production of transgenic strawberries by temporary immersion bioreactor system and verification by TAIL-PCR. BMC Biotechnol 7:1–11

Hardegger M, Sturm A (1998) Transformation and regeneration of carrot (*Daucus carota* L.). Mol Breed 4:119–127

Harwood WA (2012) Advances and remaining challenges in the transformation of barley and wheat. J Exp Bot 63:1791–1798

He DG, Mouradov A, Yang YM, Mouradova E, Scott KJ (1994) Transformation of wheat (*Triticum aestivum* L.) through electroporation of protoplasts. Plant Cell Rep 14:192–196

He Z, Qiman H, Jin SU (2010) Development of alfalfa (*Medicago sativa* L.) regeneration system and *Agrobacterium*-mediated genetic transformation. Agric Sci China 9:170–178

Heeres P, Schippers-Rozenboom M, Jacobsen E, Visser RGF (2002) Transformation of a large number of potato varieties: genotype-dependent variation in efficiency and somaclonal variability. Euphytica 124:13–22

Hensel G, Valkov V, Middlefell-Williams J, Kumlehn J (2007) Efficient generation of transgenic barley: the way forward to modulate plant-microbe interactions. J Plant Physiol 165:71–82

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282

Hoa TTC, Bong BB (2003) Efficient *Agrobacterium*-mediated transformation of indica rice (*Oryza sativa* L.) using mannose selection system. Vietn J Agric Rural Dev 1+2:60–63

Hoekstra S, van Bergen S, Brouwershaven IR, Schilperoot RA, Heidekamp F, van der Mark F (1996) The interaction of 2,4-D application and mannitol pretreatment in anther and microspore culture of *Hordeum vulgare* L. cv. Igri J Physiol 148:696–700

Holm PB, Olsen O, Schnorf M, Brinch-Pederse H, Knudsen S (2000) Transformation of barley by microinjection into isolated zygote protoplasts. Transgenic Res 9:21–32

Holme IB, Brinch-Pedersen H, Lange M, Holm PB (2008) Transformation of different barley (*Hordeum vulgare* L.) cultivars by *Agrobacterium tumefaciens* infection of in vitro cultured ovules. Plant Cell Rep 27:1833–1840

Hu L, Li Y, Xu W, Zhang G, Zhang L, Qi X, Dong H (2012) Improvement of the photosynthetic characteristics of transgenic wheat plants by transformation with the maize C4 phosphoenolpyruvate carboxylase gene. Plant Breed 131:385–391

Hu T, Metz S, Chay C, Zhou HP, Biest N, Chen G, Cheng M, Feng X, Radionenko M, Lu F, Fry J (2003) *Agrobacterium*-mediated largescale transformation of wheat (*Triticum aestivum* L.) using glyphosate selection. Plant Cell Rep 21:1010–1019

Huang CN, Wang JH, Yan QS, Zhang SQ, Yan QF (1995) Plant regeneration from rice (*Oryza sativa* L.) embryogenic suspension cell cryopreserved by vitrification. Plant Cell Rep 14:730–734
Huang X, Huang X-L, Xiao W, Zhao J-T, Dai X-M, Chen Y-F, Li X-J (2007) Highly efficient *Agrobacterium*-mediated transformation of embryogenic cell suspensions of Musa acuminate cv. Mas (AA) via a liquid co-cultivation system. Plant Cell Rep 26:1755–1762
Ignacimuthu S, Arockiasamy S, Terada R (2000) Genetic transformation of rice: current status and future prospects. Curr Sci 79:186–195
Ijaz S, Rana IA, Khan IA, Saleem M (2012) Establishment of an *in vitro* regeneration system for genetic transformation of selected sugarcane genotypes. Genet Mol Res 11:512–530
Ilic-Grubor K, Attree SM, Fowke LC (1998) Induction of microspore-derived embryos of Brassica napus L. with polyethylene glycol (PEG) as osmoticum in a low sucrose medium. Plant Cell Rep 17:329–333
Indrianto A, Heberle-Bors E, Touraev A (1999) Assessment of various stresses and carbohydrates for their effect on the induction of embryogenesis in isolated wheat microspores. Plant Sci 143:71–79
Ishigaki G, Gondo T, Suenaga K, Akashi R (2012) Fertile transgenic *Brachiaria ruziziensis* (ruzigrass) plants by particle bombardment of tetraploidized callus. J Plant Physiol 169:546–549
Ishikawa S, Bang SW, Kaneko Y, Matsuzawa Y (2003) Production and characterization of intergeneric somatic hybrids between *Moricandia arvensis* and *Brassica oleracea*. Plant Breed 122:233–238
Ivic SD, Smigocki AC (2003) Transformation of sugar beet cell suspension cultures. In Vitro Cell Dev Biol Plant 39:573–577
Jähne A, Lörz H (1995) Cereal microspore culture. Plant Sci 109:1–12
Jain RK, Jain S, Wang B, Wu R (1996) Optimization of biolistic method for transient gene expression and production of agronomically useful transgenic Basmati rice plants. Plant Cell Rep 15:963–968
Jardinaud M-F, Souvré A, Alibert G (1993) Transient GUS gene expression in *Brassica napus* electroporated microspores. Plant Sci 93:177–184
Jha AK, Dahleen LS, Suttle JC (2007) Ethylene influences green plant regeneration from barley callus. Plant Cell Rep 26:285–290
Jimenez VM (2001) Regulation of in vitro somatic embryogenesis with emphasis on the role of endogenous hormones. Rev Bras Fisiol Veg 13:196–223
Joersbo M, Brunstedt J (1990) Direct gene transfer to plant protoplasts by mild sonication. Plant Cell Rep 9:207–210
Jones HD (2005) Wheat transformation: current technology and applications to grain development and composition. J Cereal Sci 41:137–147
Jones HW, Doherty A, Wu H (2005) Review of methodologies and a protocol for the *Agrobacterium-mediated* transformation of wheat. Plant Methods 1:5–11
Jones-Villeneuve E, Huang B, Prudhomme I, Bird S, Kemble R, Hattori J (1995) Assessment of microinjection for introducing DNA into uninuclear microspores of rapeseed. Plant Cell Tiss Org Cult 40:97–100
Kaeppler HE, Gu W, Somers DA, Rines HW, Cockburn AF (1990) Silicon carbide fiber-mediated DNA delivery into plant cells. Plant Cell Rep 8:415–418
Kahrizi D (2009) Study of androgenesis and spontaneous chromosome doubling in barley (*Hordeum vulgare* L.) advanced line using isolated microspore culture. Int. J. Plant Breed 3:111–114
Kahrizi D, Mohammadi R (2009) Study of androgenesis and spontaneous chromosome doubling in Barley (*Hordeum vulgare* L.) genotypes using isolated microspore culture. Acta Agron Hun 57:155–164
Kalla R, Chu P, Spangenberg G (2001) Moleular breeding of forage legumes for virus resistance. In: Spangenberg G (ed) Molecular breeding of forage and turf. Kluwer, Dordrecht, pp 219–237
Kamo K, Blowers A, Smith F, Lawson R (1995) Stable transformation of *Gladiolus* using suspension cells and callus. J Am Soc Hortic Sci 120:347–352
Kao NN, Michayluk MR (1975) Nutritional requirement for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126:105–110

Kasha KJ, Simion E, Oro R, Yao QA, Hu TC, Carlson AR (2001) An improved *in vitro* technique for isolated microspore culture of barley. Euphytica 120:379–385

Khanna HK, Daggard GE (2003) *Agrobacterium* tumefaciens-mediated transformation of wheat using a superbinary vector and a polyamine-supplemented regeneration medium. Plant Cell Rep 21:429–436

Khanna H, Becker D, Kleidon J, Dale J (2004) Centrifugation Assisted *Agrobacterium* tumefaciens-mediated Transformation (CAAT) of embryogenic cell suspensions of banana (*Musa* spp. Cavendish AAA and Lady finger AAB). Mol Breed 4:239–252

Kim JS, Lee SY, Park SU (2008) Resveratrol production in hairy root culture of peanut. *Arachis hypogaea* L. transformed with different *Agrobacterium rhizogenes* strains. Afr J Biotechnol 7:3788–3790

Kim JS, Lee SY, Eom SH, Park SU (2010) Improved shoot organogenesis and plant regeneration of *Echinacea angustifolia* DC. J Med Plants Res 4:587–591

Klein TM, Gradziel T, Fromm ME, Sanford JC (1988a) Factors influencing gene delivery into Zea maize cells by high-velocity microprojectiles. Bio/Technol 6:559–563

Klein TM, Harper EC, Svab Z, Sanford JC, Fromm ME, Maliga P (1988b) Stable genetic transformation of intact *Nicotiana* cells by the particle bombardment process. Proc Natl Acad Sci 85:8502–8505

Klein TM, Wolf ED, Wu R, Sanford JC (1987) High-velocity microprojectiles for delivering nucleic acids into living cells. Nature 327:70–73

Kumar S, Bhat V (2012) High-frequency direct plant regeneration via multiple shoot induction in the apomictic forage grass *Cenchrus ciliaris* L. In Vitro Cell Dev Biol Plant 48:241–248

Kurmi US, Sharma DK, Tripathi MK, Tiwari R, Baghel BS, Tiwari S (2011) Plant regeneration of *Vitis vinifera* (L) via direct and indirect organogenesis from cultured nodal segments. J Agric Technol 7:721–737

Lazzeri PA, Jones HD (2009) Transgenic wheat, barley and oats: Production and characterization. Methods Mol Biol 478:3–20

Lee S-H, Lee D-G, Woo H-S, Lee K-W, Kim D-H, Kwak S-S, Kim J-S, Kim H-G, Ahsan N, Choi MS, Yang J-K, Lee B-H (2006) Production of transgenic orchardgrass via *Agrobacterium-mediated* transformation of seed-derived callus tissues. Plant Sci 171:408–414

Li J, Ye X, An B, Du L, Xu H (2012) Genetic transformation of wheat: current status and future prospects. Plant Biotechnol Rep 6:183–193

Li Q, Robson PRH, Bettany AJE, Donnison IS, Thomas H, Scott IM (2004) Modification of senescence in ryegrass transformed with IPT under the control of a monocot senescence-enhanced promoter. Plant Cell Rep 22:816–821

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18:100–127

Lionneton E, Beuret W, Delaitre C, Ochatt S, Rancillac M (2001) Improved microspore culture and double haploid plant regeneration for the brown condiment mustard (*Brassica juncea*). Plant Cell Rep 20:126–30

Liu W, Zheng M, Konzak CF (2002a) Improving green plant production via isolated microspore culture in bread wheat (*Triticum aestivum* L.). Plant Cell Rep 20:821–824

Liu W, Zheng M, Polle E, Konzak CF (2002b) Highly efficient doubled-haploid production in wheat (*Triticum aestivum* L.) via induced microspore embryogenesis. Crop Sci 42:686–692

Lloyd G, McCown B (1980) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Proc Int Plant Prop Soc 30:421–427

Loganathan M, Maruthasalam S, Shiu LY, Lien WC, Hsu WH, Lee PF, Yu CW, Lin CH (2010) Regeneration of soybean (*Glycine max* L. Merrill) through direct somatic embryogenesis from the immature embryonic shoot tip. In Vitro Cell Dev Biol Plant 46:265–273

Lucca P, Ye X, Potrykus I (2001) Effective selection and regeneration of transgenic rice plants with mannose as selective agent. Mol Breed 7:43–49

Luthra R, Varsha V, Dubey RK, Srivastava AK, Kumar S (1997) Microprojectile mediated plant transformation: a bibliographic research. Euphytica 95:269–294

Maheshwari P, Selvaraj G, Kovalchu I (2011) Optimization of *Brassica napus* (canola) explant regeneration for genetic transformation. New Biotechnol 29(1):144–155

Maju TT, Soniya EV (2012) *In vitro* regeneration system for multiplication and transformation in *Piper nigrum* L. Int J Med Arom Plants 2:178–184

Matsumoto K, Vilarinhos AD, Oka S (2002) Somatic hybridization by electrofusion of banana protoplasts. Euphytica 125:317–324

McCabe DE, Swain WF, Martinell BJ, Christou P (1988) Stable transformation of soybean (*Glycine max*) by particle acceleration. Biotechnology 6:923–926

Mejza SJ, Morgant V, DiBona DE, Wong JR (1993) Plant regeneration from isolated microspores of *Triricum aestivum*. Plant Cell Rep 2:149–153

Mendes BMJ, Mourao FD, Filho AO, Farias PC, De M, Benedito VA (2001) Citrus somatic hybridization with potential for improved blight and CTV resistance. In Vitro Cell Dev Biol Plant 37:490–495

Menges M, Murray JAH (2004) Cryopreservation of transformed and wild-type *Arabidopsis* and tobacco cell suspension cultures. Plant J 37:635–644

Mentewab A, Letellier V, Marque C, Sarrafi A (1999) Use of anthocyanin biosynthesis stimulatory genes as markers for the genetic transformation of haploid embryos and isolated microspores in wheat. Cereal Res Commun 27:17–24

Ming-zhi L (1996) Promotion of carrot suspension cell transformation and plant regeneration by *Agrobacterium* harbouring binary vector pretreated with phenolic compounds. Acta Bot Sin 38:203–208

Mitić N, Dodig D, Nikolić R, Ninković S, Vinterhalter D, Vinterhalter B (2009) Effects of donor plant environmental conditions on immature embryo cultures derived from worldwide origin wheat genotypes. Russ J Plant Physiol 56:540–545

Montague A, Ziauddin A, Lee R, Ainley WM, Strommer J (2007) High-efficiency phosphinothricin-based selection for alfalfa transformation. Plant Cell Tiss Org Cult 91:29–36

Moukadiri O, Lopes CR, Cornejo MJ (1999) Physiological and genomic variations in rice cells recovered from direct immersion and storage in liquid nitrogen. Physiol Plant 105:442–449

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nada S, Chennareddy S, Goldman S, Rudrabhatla S, Potlakayala SD, Josekutty P, Deepkamal K (2011) Direct shoot bud differentiation and plantlet regeneration from leaf and petiole explants of *Begonia tuberhybrida*. Hortic Sci 46:759–764

Nakamura T, Ishikawa M (2006) Transformation of suspension cultures of bromegrass (*Bromus inermis*) by *Agrobacterium tumefaciens*. Plant Cell Tiss Org Cult 84:293–299

Nandadeva YL, Lupi CG, Meyer CS, Devi PS, Potrykus I, Bilang R (1999) Microprojectile-mediated transient and integrative transformation of rice embryogenic suspension cells: effects of osmotic cell conditioning and of the physical configuration of plasmid DNA. Plant Cell Rep 18:500–504

Nash T-D, Davies ME (1972) Some aspects of growth and metabolism of Paul's Scarlet rose cell suspensions. J Exp Bot 23:75–91

Nehlin L, Möllers C, Bergman P, Glimelius K (2000) Transient *β-gus* and *gfp* gene expression and viability analysis of microprojectile bombarded microspores of *Brassica napus* L. J Plant Physiol 156:175–183

Neuhaus G, Spangenberg G, Scheid OM, Schweiger HG (1987) Transgenic rapeseed plants obtained by the microinjection of DNA into microspore-derived embryoids. Theor Appl Genet 75:30–36

Newell CA (2000) Plant transformation technology: developments and applications. Mol Biotechnol 16:53–65

Niaz F, Quraishi A (2002) Effect of growth regulators on the regeneration potential of two sugarcane cultivars SPF-213 and CPF-237. Pak J Biol Sci 10:1081–1083

Nishimura A, Ashikari M, Lin S, Takashi T, Angles ER, Yamomoto T, Matsuoka M (2005) Isolation of a rice regeneration quantitative trait loci gene and its application to transformation systems. Proc Natl Acad Sci U S A 102:11940–11944

Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163:85–87

Nugent GD, Coyne S, Ngyuen TT, Kavanagh TA, Dix PJ (2006) Nuclear and plastid transformation of *Brassica oleracea* var. *botrytis* (cauliflower) using PEG-mediated uptake of DNA into protoplasts. Plant Sci 170:135–142

Ozawa K, Takaiwa F (2010) Highly efficient *Agrobacterium*-mediated transformation of suspension-cultured cell clusters of rice (*Oryza sativa* L.). Plant Sci 179:333–337

Pandey RN, Singh SP, Rastogi J, Sharma ML, Singh RK (2012) Early assessment of genetic fidelity in sugarcane (*Saccharum officinarum*) plantlets regenerated through direct organogenesis with RAPD and SSR markers. Aust J Crop Sci 6:618–624

Panis B, Helliot B, Strosse H, Remy S, Lepoivre P, Swennen R (2005) Germplasm conservation, virus eradication and safe storage of transformation competent cultures in banana: The importance of cryopreservation. Acta Hortic 692:51–59

Parimalan R, Venugopalan A, Giridhar P, Ravishankar GA (2011) Somatic embryogenesis and *Agrobacterium*-mediated transformation in *Bixa orellana* L. Plant Cell Tiss Org Cult 105: 317–328

Park E-H, Bae H, Park WT, Kim YB, Chae SC, Park SU (2012) Improved shoot organogenesis of gloxinia (*Sinningia speciosa*) using silver nitrate and putrescine treatment. Plant Omics J 5:6–9

Parrott WA, Hoffman LM, Hildebrand DF, Williams EG, Collins GB (1989) Recovery of primary transformants of soybean. Plant Cell Rep 7:615–617

Pasternak TP, Prinsen E, Ayaydin F, Miskolczi P, Potters G, Asard H, van Onckelen HA, Dudits D, Feher A (2002) The role of auxin, pH, and stress in the activation of embryogenic cell division in leaf protoplast-derived cells of alfalfa. Plant Physiol 129:1807–1819

Patil AS, Paikrao HM (2012) A novel regeneration system for a wild passion fruit species (*Passiflora foetida* L.) Based on direct somatic embryogenesis from leaf explants. Global J Res Med Plants Indigen Med 1:485–495

Patnaik D, Khurana P (2003) Genetic transformation of Indian bread (*T. aestivum*) and pasta (*T. durum*) wheat by particle bombardment of mature embryo-derived calli. BMC Plant Biol 3:1–11

Paul A, Thapa G, Basu A, Mazumdar P, Kalita MK, Sahoo L (2010) Rapid plant regeneration, analysis of genetic fidelity and essential aromatic oil content of micropropagated plants of patchouli, *Pogostemon cablin* (Blanco) Benth. An industrially important aromatic plant. Ind Crops Prod 32:366–374

Pellegrineschi A, Brito RM, McLean S, Hoisington D (2004) Effect of 2, 4-dichlorophenoxyacetic acid and NaCl on the establishment of callus and plant regeneration in durum and bread wheat. Plant Cell Tiss Org Cult 77:245–250

Pellegrineschi A, Noguera LM, Skovmand B, Brito RM, Velazquez L, Salgado MM, Hernandez R, Warburton M, Hoisington D (2002) Identification of highly transformable wheat genotypes for mass production of fertile transgenic plants. Genome 45:421–430

Perl A, Saad S, Sahar N, Holland D (1995) Establishment of long-term embryogenic cultures of seed-less *Vitis vinifera* cultivars - a synergistic effect of auxins and the role of abscisic acid. Plant Sci 104:193–200

Piqueras A, Alburquerque N, Folta KM (2010) Explants used for the generation of transgenic plants. In: Kole C, Michler CH, Abbott AA, Hall TC (eds) Transgenic crop plants. Springer, Berlin, pp 31–56

Piqueras A, Debergh PC (1999) Morphogenesis in micropropagation. In: Soh WY, Bhojwani SS (eds) Morphogenesis in plant tissue cultures. Kluwer Acad Publ, Dordrecht, The Netherlands, pp 443–462

Popelka JC, Altpeter F (2003) Evaluation of rye (*Secale cereale* L.) inbred lines and their crosses for tissue culture response and stable genetic transformation of homozygous rye inbred line L22 by biolistic gene transfer. Theor Appl Genet 107:583–590

Potrykus I, Harms CT, Lörz H (1979) Callus formation from cell culture protoplasts of corn (*Zea mays*). Theor Appl Genet 54:209–214

Potrykus I, Saul MW, Petruska J, Paszkowski J, Shillito RD (1985) Direct gene transfer to cells of a graminaceous monocot. Mol Genet 199:183–188

Puonti-Kaerlas J, Li HQ, Sautter C, Potrykus I (1997) Production of transgenic cassava (*Manihot esculaenta* Crantz) via organogenesis and *Agrobacterium*-mediated transformation. Afr J Root Tuber Crops 2:181–186

Purkayastha J, Sugla T, Paul A, Solleti SK, Mazumdar P, Basu A, Mohommad A, Ahmed Z, Sahoo L (2010) Efficient *in vitro* plant regeneration from shoot apices and gene transfer by particle bombardment in *Jatropha curcas*. Biol Plant 54:13–20

Raemakers CJJM, Sofiari E, Taylor N, Henshaw G, Jacobsen E, Visser RGF (1996) Production of transgenic cassava (*Manihot esculenta* Crantz) plants by particle bombardment using luciferase activity as selection marker. Mol Breed 2:339–349

Raghu AV, Geetha SP, Martin G, Balachandran I, Mohanan KV (2010) Micropropagation of *Tribulus terestris* Linn. Indian J Nat Prod Res 1:232–235

Rajasekaran K, Hudspeth RL, Cary JW, Anderson DM, Cleveland TE (2000) High-frequency stable transformation of cotton (*Gossypium hirsutum* L.) by particle bombardment of embryogenic cell suspension cultures. Plant Cell Rep 19:539–545

Rajasekaran K, Grula JW, Hudspeth RL, Pofelis S, Anderson DM (1996) Herbicide-resistant Acala and Coker cottons transformed with a native gene encoding mutant forms of acetohydroxyacid synthase. Mol Breed 2:307–319

Rech EL, Vianna GR, Aragao FJL (2008) High-efficiency transformation by biolistics of soybean, common bean and cotton transgenic plants. Nat Protoc 3:410–418

Reinert J, Yeoman MM (1982) Plant cell and tissue culture: a laboratory manual. Springer, Berlin, pp. 83

Risacher T, Craze M, Bowden S, Paul W, Barsby T (2009) Highly efficient *Agrobacterium-mediated* transformation of wheat via in planta inoculation. In: Jones HD, Shewry PR (eds) Methods in molecular biology, transgenic wheat, barley and oats, vol. 478. Humana, New Jersey, pp 115–124

Ritala A, Mannonen L, Aspegren K, Salmenkallio-Marttila M, Kurtén U, Hannus R, Mendez LJ, Teeri TH, Kauppinen V (1993) Stable transformation of barley tissue culture by particle bombardment. Plant Cell Rep 12:435–440

Roby C, Martin J-B, Bligny R, Douce R (1987) Biochemical changes during sucrose deprivation in higher plant cells. Phosphorus-31 nuclear magnetic resonance studies. J Biol Chem 262: 5000–5007

Rosellini R, Capomaccio S, Ferradini N, Sardaro MLS, Nicolia A, Veronesi F (2007) Non-antibiotic, efficient selection for alfalfa genetic engineering. Plant Cell Rep 26:1035–1044

Santacruz GAA, Cruz QR, Gómez BM, González RGG, Olvera LG, Bremont JFJ, Gallegos SA, Moya EG (2009) Genetic transformation of blue grama grass with the rolA gene from *Agrobacterium rhizogenes*: regeneration of transgenic plants involves a “hairy embryo” stage. In Vitro Cell Dev Biol Plant 45:681–692

Sartoretto LM, Cid LPB, Brasileiro ACM (2002) Biolistic transformation of *Eucalyptus grandis* x *E. urophylla* callus. Funct Plant Biol 29:917–924

Sato H, Takamizo T (2006) *Agrobacterium* tumefaciens-mediated transformation of forage-type perennial ryegrass (*Lolium perenne* L.). Grassland Sci 52:95–98

Sato T, Asaka D, Harada T, Matsukawa I (1993) Plant regeneration from protoplasts of adzuki bean (*Vigna angularis* Ohwi & Ohashi). Jpn J Breed 43:183–191

Schenk RU, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Bot 50:199–204

Schnorf M, Neuhaus-Url G, Galli A, Lida S, Potrykus I, Neuhaus G (1991) An imrprovement method for transformatuion of plant cells by microinjection; molecular and genetic analysis. Transgenic Res 1:23–30

Schöpke C, Taylor N, Carcamo R, Konan NK, Marmey P, Henshaw GG, Beachy R, Fauquet C (1996) Regeneration of transgenic cassava plants (*Manihot esculenta* Crantz) from microbombarded embryogenic suspension cultures. Nat Biotechnol 14:731–735

Schreuder MM, Raemakers CJJM, Jacobsen E, Visser RGF (2001) Efficient production of transgenic plants by *Agrobacterium*-mediated transformation of cassava (*Manihot esculenta* Crantz). Euphytica 120:35–42

Schulze J (2007) Improvements in cereal tissue culture by thidiazuron: a review. Fruit Veg Cereal Sci Biotechnol 1:64–79

Schulze J, Balko C, Zener B, Koprek T, Hänsch R, Nerlich A, Mendel RR (1995) Biolistic transformation of cucumber using embryogenic suspension cultures: long-term expression of reporter genes. Plant Sci 112:197–206

Scott RJ, Draper J (1987) Transformation of carrot tissues derived from proembryogenic suspension cells: A useful model system for gene expression studies in plants. Plant Mol Biol 8:265–274

Shariatpanahi ME, Bal U, Heberle-Bors E, Touraev A (2006) Stresses applied for the re-programming of plant microspores towards *in vitro* embryogenesis. Physiol Plantarum 127:519–534

Sharma S, Sarkar D, Pandey SK, Chandel P, Tiwari JK (2011) Stoloniferous shoot protoplast, an efficient cell system in potato for somatic cell genetic manipulations. Sci Hortic 128:84–91

Sharon M, Sharan M, Castello MC (2012) *In vitro* culture studies of *Bixa orellana* L: I - differential requirements for plant regeneration from hypocotyl, leaf, cotyledonary leaf and root explants. Eur J Exp Biol 2:142–150

Sheen J (2001) Signal transduction in maize and *Arabidopsis* mesophyll protoplasts. Plant Physiol 127:1466–1475

Siddique I, Anis M (2009) Direct plant regeneration from nodal explants of *Balanites aegyptiaca* L. (Del.): a valuable medicinal tree. New Forests 37:53–62

Sinha A, Wetten AC, Caligari PDS (2003) Effect of biotic factors on the isolation of *Lupinus albus* protoplasts. Aust J Bot 51:103–109

Smith RH (2012) Plant tissue culture: techniques and experiments, 3rd ed. pp. 188. Academic, USA

Somers RDA, Rines H, Weining GU, Kaeppler HF, Bushnell WR (1992) Fertile transgenic oat plants. Biotechnology 10:1589–1594

Spangenberg G, Wang ZY, Wu XL, Nagel J, Iglesias VA, Potrykus I (1995a) Transgenic tall fescue (*Festuca arundinacea*) and red fescue (*F. rubra*) plants from microprojectile bombardment of embryogenic suspension cells. J Plant Physiol 145:693–701

Spangenberg G, Wang ZY, Wu X, Nagel J, Potrykus I (1995b) Transgenic perennial ryegrass (*Lolium perenne*) plants from microprojectile bombardment of embryogenic suspension cells. Plant Sci 108:209–217

Sparks CA, Jones HD (2009) Biolistics transformation of wheat. In: Jones HD, Shewry PR (eds) Methods in molecular biology, transgenic wheat, barley and oats, vol. 478. Humana, New Jersey, pp 71–92

Stöger E, Fink C, Pfosser M, HeberleBors E (1995) Plant transformation by particle bombardment of embryogenic pollen. Plant Cell Rep 14:273–278

Stojakowska A, Malarz J, Szewczyk A, Kisiel W (2012) Caffeic acid derivatives from a hairy root culture of *Lactuca virosa*. Acta Physiol Plant 34:291–298

Street, H. E. 1973. Plant tissue culture. Botanical monographs, vol. 11. University of California Press, California. Blackwell Scientific Publications, Berkeley. pp. 507

Su YH, Liu YB, Zhang XS (2011) Auxin-cytokinin interaction regulates meristem development. Mol Plant 4:616–625

Sujatha M, Vijay S, Vasavi S, Reddy PV, Rao SC (2012) *Agrobacterium* mediated transformation of cotyledons of mature seeds of multiple genotypes of sunflower (*Helianthus annuus* L.). Plant Cell Tiss Org Cult 110:275–287

Supartana P, Shimizu T, Nogawa M, Shioiri H, Nakajima T, Haramoto N, Nozue M, Kojima M (2006) Development of simple and efficient in planta transformation method for wheat (*Triticum aestivum* L.) using *Agrobacterium* tumefaciens. J Biosci Bioeng 102:162–170

Szechyńska-Hebda M, Skrzypek E, Dabrowska G, Kościelniak J, Filek M, Wędzony M (2007) The role of oxidative stress induced by growth regulators in the regeneration process of wheat. Acta Physiol Plant 29:327–337

Szechyńska-Hebda M, Skrzypek E, Dąbrowska G, Wędzony M, van Lammeren A (2012) The effect of endogenous hydrogen peroxide induced by cold treatment in the improvement of tissue regeneration efficiency. Acta Physiol Plant 34:547–560

Taparia Y, Gallo M, Altpeter F (2012) Comparison of direct and indirect embryogenesis protocols, biolistic gene transfer and selection parameters for efficient genetic transformation of sugarcane. Plant Cell Tiss Org Cult 111:131–141

Touraev A, Heberle-Bors E (1999) Microspore embryogenesis and *in vitro* pollen maturation in tobacco. In: Hall R (ed) Plant cell culture protocols. Humana, New Jersey, pp 281–291

Touraev A, Forster BP, Jain SM (2009) Advances in haploid production in higher plants. Springer Science + Business Media B.V, USA, pp. 348

Touraev A, Indrianto A, Wratschko I, Vicente O, HeberleBors E (1996a) Efficient microspore embryogenesis in wheat (*Triticum aestivum* L.) induced by starvation at high temperature. Sex Plant Reprod 9:209–215

Touraev A, Pfosser M, Vicente O, HeberleBors E (1996b) Stress as the major signal controlling the developmental fate of tobacco microspores: towards a unified model of induction of microspore/pollen embryogenesis. Planta 200:144–152

Travella S, Ross SM, Harden J, Everett C, Snape JW, Harwood WA (2005) A comparison of transgenic barley lines produced by particle bombardment and *Agrobacterium* mediated techniques. Plant Cell Rep 23:780–789

Trick HN, Finer JJ (1998) Sonicationassisted *Agrobacterium* mediated transformation of soybean [*Glycine max* (L.) Merrill] embryogenic suspension culture tissue. Plant Cell Rep 17:482–488

Turhan H (2004) Callus induction and growth in transgenic potato genotypes. Afr J Biotechnol 3:375–378

Tyagi N, Dahleen LS, Bregitzer P (2010) Candidate genes within tissue culture regeneration QTL revisited with a linkage map based on transcript derived markers. Crop Sci 50:1697–1707

Urushibara S, Tozawa Y, KawagishiKobayashi M, Wakasa K (2001) Efficient transformation of suspension-cultured rice cells mediated by *Agrobacterium* tumefaciens. Breed Sci 5(33):38

Varshney A, Kant T, Sharma VK, Rao A, Kothari SL (1996) High frequency plant regeneration from immature embryo cultures of *Triticum aestivum* and *T. durum*. Cereal Res Commun 24:409–416

Vidal JR, Kikkert JR, Donzelli BD, Wallace PG, Reisch BI (2006) Biolistic transformation of grapevine using minimal gene cassette technology. Plant Cell Rep 25:807–814

Vishnudasan D, Tripathi MN, Rao U, Khurana P (2005) Assessment of nematode resistance in wheat transgenic plants expressing potato proteinase inhibitor (PIN2) gene. Transgenic Res 14:665–675

Wang B, Zhang Z, Yin Z, Feng C, Wang Q (2012) Novel and potential application of cryopreservation to plant genetic transformation. Biotechnol Adv 30:604–612

Wang K, Drayton P, Frame B, Dunwell J, Thompson J (1995) Whiskermediated plant transformation: an alternative technology. In Vitro Cell Dev Biol 31:101–104

Wang M, Hoekstra S, van Bergen S, Lamers GEM, Oppedijk BJ, van der Heiden MW, de Priester W, Schilperoort RA (1999) Apoptosis in developing anthers and the role of ABA in this process during androgenesis in *Hordeum vulgare* L. Plant Mol Biol 39:489–501

Wang Q, Li P, Hanania U, Sahar N, Mawassi M, Gafny R, Sela I, Tanne E, Perl A (2005) Improvement of *Agrobacterium* mediated transformation efficiency and transgenic plant regeneration of *Vitis vinifera* L. by optimizing selection regimes and utilizing cryopreserved cell suspensions. Plant Sci 168:565–571

Wang ZY, Ge Y (2005) *Agrobacterium* mediated high efficiency transformation of tall fescue (*Festuca arundinacea* Schreb.). J Plant Physiol 162:103–113

Wang ZY, Bell J, Lehmann D (2004) Transgenic Russian wildrye (*Psathyrostachys juncea*) plants obtained by biolistic transformation of embryogenic suspension cells. Plant Cell Rep 22:903–909

Weber S, Friedt W, Landes N, Molinier J, Himber C, Rousselin P, Hahne G, Horn R (2003) Improved Agrobacterium mediated transformation of sunflower (*Helianthus annuus* L.): assessment of macerating enzymes and sonication. Plant Cell Rep 21:475–82

Wędzony M, Forster BP, Zur I, Golemiec E, Szechyńska-Hebda M, Dubas E, Gołębiowska G (2009) Progress in doubled haploid technology in higher plants. In: Touraev A, Jain M, Forster B (eds) Advances in haploid production in higher plants. Springer Science + Business Media B.V, USA, p 133

Weir B, Gu X, Wang M, Upadhyaya N, Elliott AR, Brettell RIS (2001) *Agrobacterium* tumefaciensmediated transformation of wheat using suspension cells as a model system and green fluorescent protein as a visual marker. Aust J Plant Physiol 28:807–818

Winkelmann T, Specht J, Serek M (2006) Efficient plant regeneration from protoplasts isolated from embryogenic suspension cultures of *Cyclamen persicum* Mill. Plant Cell Tiss Org Cult 86:337–347

Wright E, Dixon RA, Wang ZY (2006) Medicago truncatula transformation using cotyledon explants. In: Wang K (ed) *Agrobacterium* protocols, vol. 2, vol 2. Humana, New Jersey, pp 129–135

Wright M, Dawson J, Dunder E, Suttie J, Reed J, Kramer C, Chang Y, Novitzky R, Wang H, ArtimMoore L (2001) Efficient biolistic transformation of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) using the phosphomannose isomerase gene pmi, as the selectable marker. Plant Cell Rep 20:429–436

Wu HX, Doherty A, Jones HD (2008) Efficient and rapid *Agrobacterium* mediated genetic transformation of durum wheat (*Triticum turgidum* L. var. Durum) using additional virulence genes. Trans Res 17:425–436

Wu H, Doherty A, Jones HD (2009) *Agrobacterium* mediated transformation of bread and durum wheat using freshly isolated immature embryos. Methods Mol Biol 478:93–103

Wu H, McCormac AC, Elliott MC, Chen DF (1998) *Agrobacterium* mediated stable transformation of cell suspension cultures of barley (*Hordeum vulgare*). Plant Cell Tiss Org Cult 54:161–171

Wu YY, Chen QJ, Chen M, Chen J, Wang XC (2005) Salttolerant transgenic perennial ryegrass (*Lolium perenne* L.) obtained by *Agrobacterium* tumefaciensmediated transformation of the vacuolar Na+/H+ antiporter gene. Plant Sci 169:65–73

Wu YY, Chen QJ, Cui XH, Chen H, Chen J, Wang XC (2007) Efficient regeneration and *Agrobacterium* mediated stable transformation of perennial ryegrass. Russ J Plant Physiol 54:524–529

Xi M, Fang L, Qiu S, Lu Y, Shi J (2012) A high efficiency regeneration system of oriental lily cultivar 'Constanta'. Mol Plant Breed 3:115–120

Xi Y, Fu C, Ge Y, Nandakumar R, Hisano H, Bouton J, Wang ZY (2009) *Agrobacterium* mediated transformation of switchgrass and inheritance of the transgenes. Bioenerg Res 2:275–283

Xie DY, Sharma SB, Wright E, Wang ZY, Dixon RA (2006) Metabolic engineering of proanthocyanidins through coexpression of anthocyanidin reductase and the PAP1 MYB transcription factor. Plant J 45:895–907

Yang JL, Seong ES, Kim MJ, Ghimire BK, Kang WH, Yu CY, Li CH (2010) Direct somatic embryogenesis from pericycle cells of broccoli (*Brassica oleracea* L. var. *italica*) root explants. Plant Cell Tiss Org Cult 100:49–58

Yang J, Bi HP, Fan WJ, Zhang M, Wang HX, Zhang P (2011) Efficient embryogenic suspension culturing and rapid transformation of a range of elite genotypes of sweet potato (*Ipomoea batatas* [L.] Lam.). Plant Sci 181:701–711

Yao JL, Wu JH, Gleave AP, Morris BAM (1996) Transformation of citrus embryogenic cells using particle bombardment and production of transgenic embryos. Plant Sci 113:175–183

Yao QA, Simion E, William M, Krochko J, Kasha KJ (1997) Biolistic transformation of haploid isolated microspores of barley (*Hordeum vulgare* L.). Genome 40:570–581

Ye X, Wang ZY, Wu X, Potrykus I, Spangenberg G (1997) Transgenic Italian ryegrass (*Lolium multiflorum*) plants from microprojectile bombardment of embryogenic suspension cells. Plant Cell Rep 16:379–384

Yoshimatsu K (2008) Tissue culture of medicinal plants: micropropagation, transformation and production of useful secondary metabolites. Studies in natural products chemistry. Bioactive Nat Prod (Part N) 34:647–752

Yue SJ, Li H, Li YW, Zhu YF, Guo JK, Liu YJ, Chen Y, Jia X (2008) Generation of transgenic wheat lines with altered expression levels of 1Dx5 highmolecular weight glutenin subunit by RNA interference. J Cereal Sci 47:153–161

Zale JM, Agarwal S, Loar S, Steber CM (2009) Evidence for stable transformation of wheat by floral dip in *Agrobacterium* tumefaciens. Plant Cell Rep 28:903–913

Zang N, Zhai H, Gao S, Chen W, He S, Liu Q (2009) Efficient production of transgenic plants using the bar gene for herbicide resistance in sweet potato. Sci Hortic 122:649–653

Zhang HC, Liu JM, Lu HY, Gao SL (2009) Enhanced flavonoid production in hairy root cultures of *Glycyrrhiza uralensis* Fisch by combining the overexpression of chalcone isomerase gene with the elicitation treatment. Plant Cell Rep 28:1205–1213

Zhang HM, Yang H, Rech EL, Golds TJ, Davis AS, Mulligan BJ, Cocking EC, Davey MR (1988) Transgenic rice plants produced by electroporation-mediated plasmid uptake into protoplasts. Plant Cell Rep 7:379–384

Zhang JY, Broeckling CD, Blancaflor EB, Sledge MK, Sumner LW, Wang ZY (2005) Overexpression of WXPI, a putative *Medicago truncatula* AP2 domain-containing transcription factor gene, increases cuticular wax accumulation and enhances drought tolerence in transgenic alfalfa (*Medicago sativa*). Plant J 42:689–707

Zhao J, Cui J, Liu J, Liao F, Henny RJ, Chen J (2012a) Direct somatic embryogenesis from leaf and petiole explants of *Spathiphyllum* 'Supreme' and analysis of regenerants using flow cytometry. Plant Cell Tiss Organ Cult 110:239–249

Zhao J, Zhang Q, Xie J, Hung C, Cui J, Henny RJ, Chen J (2012b) Plant regeneration via direct somatic embryogenesis from leaf and petiole explants of *Epipremnum aureum* 'Marble Queen' and characterization of selected variants. Acta Physiol Plant 34:1461–1469

Zhao JP, Simmonds DH, Newcomb W (1996a) High frequency production of doubled haploid plants of *Brassica napus* cv. Topas derived from colchicineinduced microspore embryogenesis without heat shock. Plant Cell Rep 15(668):671

Zhao JP, Simmonds DH, Newcomb W (1996b) Induction of embryogenesis with colchicine instead of heat in microspores of *Brassica napus* L. cv. Topas. Planta 198:433439

Zhao TJ, Zhao SY, Chen HM, Zhao QZ, Hu ZM, Hou BK, Xia GM (2006) Transgenic wheat progeny resistant to powdery mildew generated by *Agrobacterium* inoculum to the basal portion of wheat seedlings. Plant Cell Rep 25:1199–1204

Zheng H, Wang L, Cheng A, Liu C (2003) Electrofusion of tobacco protoplasts in space. Chin Sci Bull 48:18196–71970

Zheng MY, Weng Y, Liu W, Konzak CF (2001) The effect of ovaryconditioned medium on microspore embryogenesis in common wheat (*Triticum aestivum* L.). Plant Cell Rep 20:802–807

Zhou WJ, Hagberg P, Tang GX (2002a) Increasing embryogenesis and doubling efficiency by immediate colchicines treatment of isolated microspores in spring Brassica napus. Euphytica 128:27–34

Zhou WJ, Tang GX, Hagberg P (2002b) Efficient production of doubled haploid plants by immediate colchicine treatment of isolated microspores in winter *Brassica napus*. Plant Growth Regul 37: 185–192

Zuo J, Niu QW, Ikeda Y, Chua NH (2002) Marker free transformation: increasing transformation frequency by the use of regeneration promoting genes. Curr Opin Biotechnol 13:173–180

Zur I, Dubas E, Golemiec E, SzechyńskaHebda M, Janowiak F, Wędzony M (2008) Stressinduced changes important for effective androgenic induction in isolated microspore culture of triticale (×*Triticosecale* Wittm.). Plant Cell Tiss Org Cult 94:319–328

Zur I, Dubas E, Golemiec E, SzechyńskaHebda M, Golebiowska G, Wędzony M (2009) Stressrelated variation in antioxidative enzymes activity and cell metabolism efficiency associated with embryogenesis induction in isolated microspore culture of triticale (×*Triticosecale* Wittm.). Plant Cell Rep 28:1279–1287

Chapter 4
Methods and Role of Embryo Rescue Technique in Alien Gene Transfer

Monika M. Lulsdorf, Alison Ferrie, Susan M.H. Slater, and Hai Ying Yuan

Abstract Embryo abortion occurs frequently in wide crosses, and thus embryo rescue is required for survival of the next generation. Rescues are performed by either directly transferring the excised embryo to an artificial medium or indirectly through flower (ovary), immature seed (fertilized ovule), or pod (silique) culture. Various techniques used for oil crops, cereals, legumes, and horticultural crops are presented. Altering medium components were the major routes for developing protocols for each species with adaptations to the base medium, sucrose concentration, or vitamin and growth regulator content. Monocot culture tended to be more direct than dicot culture, where many protocols required a multi-step approach from pod to ovule culture to embryo rescue, shoot regeneration, and root induction. Each step required a specific medium and growth conditions. Hybrid embryos as young as 2 days after pollination have been recovered. However, many species such as soybean and chickpea still need procedures for rescue of very young embryos. In other species hurdles such as poor rooting have been overcome by using grafting techniques. Embryo rescue remains a useful component in any breeding program where wide or interspecific crosses are preformed, where rapid cycling through generations is used, and where germplasm preservation is required.

Keywords Embryo rescue • Protocols • Oil crops • Cereals • Legumes • Horticulture crops

M.M. Lulsdorf, Ph.D. (✉) • S.M.H. Slater • H.Y. Yuan
Crop Development Centre (CDC), University of Saskatchewan, 51 Campus Drive, Saskatoon, SK, Canada S7N 5A8
e-mail: monika.lulsdorf@usask.ca

A. Ferrie
National Research Council of Canada (NRCC), Saskatoon, Canada

A. Pratap and J. Kumar (eds.), *Alien Gene Transfer in Crop Plants, Volume 1: Innovations, Methods and Risk Assessment*, DOI 10.1007/978-1-4614-8585-8_4,
© Springer Science+Business Media New York 2014

4.1 Introduction

Embryo abortion occurs frequently in crosses between a cultivated species and its wild relatives but also in wide crosses made between different species for the purpose of gene transfer or chromosome elimination. In these cases, embryo rescue is required because the embryo stops developing during various stages of seed development and removal of the embryo from the parent plant is necessary for survival. Rescue is done by directly transferring the excised embryo to an artificial medium or indirectly through flower (ovary), immature seed (fertilized ovule), or pod (silique) culture.

The causes for post-fertilization barriers are multifarious. They can occur due to chromosome alterations, ploidy differences, lack of starch availability at syngamy, poor connections between chalazal cells and the cytoplasmic envelope as observed for peanut (Pattee and Stalker 1991), differences in growth rates as reported for *Cicer* species (Ahmad and Slinkard 1991), lack of delayed or degenerating endosperm development, early suspensor degeneration, or mismatch between the different chromosome sets.

The culture medium replaces the endosperm and provides the nutrients to the developing embryo. The younger the aborting hybrid, the more complex are the steps involved in rescuing the embryos and the more complex the medium requirements. For example, Geerts et al. (2011) succeeded in rescuing 2–5-day-old bean interspecific hybrids using a six-step procedure. Since removal of young, fragile embryos frequently leads to physical damage, immature seed (ovule) or pod (silique) cultures are the preferred methods until the embryo reaches more mature stages which is generally past the critical heart-shaped stage. This chapter provides an overview of recent methods used for embryo rescue in a wide variety of species and highlights some procedures for rescue of very young embryos.

4.2 Oil Crops

4.2.1 Brassicaeae

Most of the breeding work within the *Brassica* species has been focused on resynthesis of *B. napus* or *B. juncea* in order to increase the genetic variability of these crops. For some interspecific crosses, it is relatively easy to generate hybrid seeds, but others have post-fertilization barriers that require embryo rescue. If the barrier occurs very early in hybrid embryo development, it is not technically possible to isolate the developing embryo, and methods such as culturing the silique or isolated ovaries are used. Thus, a wide variety of methods are available for embryo rescue in this family.

4.2.1.1 Embryo Culture

Nishi et al. (1959) used embryo culture methods in *Brassica* crosses with some success. A review of the literature (Inomata 1993) showed that the hybrid production rate ranged from 0 to 25.1 (number of hybrids/number of flowers pollinated). For some crosses, embryo culture has become a fairly routine method for generating interspecific and intergeneric hybrids. In other crosses, in which hybrid embryos are not easily obtained, basic studies were required to refine culture conditions and medium constituents.

Embryo survival relies on the timely removal of the embryo after pollination. This depends on the species and genotypes involved in the specific cross. Abortion usually occurs from the mid to late stages of embryogenesis in *Brassicae*. Therefore, embryos can be cultured anywhere from 6 to 28 days depending on the cross. Depending on the genotype of the maternal parent in crosses between *B. oleracea* and *B. rapa,* hybrid embryos cultured 16–20 days after pollination (DAP) showed signs of degeneration (Wen et al. 2008), whereas in other crosses, viable ovules were still present 16–22 DAP. In crosses of *B. campestris* ssp. *pekinensis* × *B. oleracea* var. *acephala*, embryos were cultured 6–20 DAP with the highest frequency of plantlet production obtained from those embryos cultured at 9–11 DAP (Zhu and Wei 2009). Other studies have shown that the highest frequency of hybrid embryos came from those rescued 20–28 DAP when employing *B. oleracea* var. *alboglabra* as the female parent (Rahman 2004) or rescued 18–22 DAP when using *B. carinata* as the female parent.

Composition of medium is a major factor for successful embryo rescue (Table 4.1). In crosses between *B. juncea* and *B. napus*, embryo culture experiments compared two basal media, MS (Murashige and Skoog 1962) or B5 (Gamborg et al. 1968), and different combinations of NAA and BAP (Zhang et al. 2003). MS medium was better than B5 in terms of callus production and regenerated plants and is generally preferred in *Brassica* embryo culture (Table 4.1) although liquid NN medium (Nitsch and Nitsch 1967) has also been used (Ripley and Beversdorf 2003). As for growth regulators, 0.1–0.3 mg/L NAA with 1.5–2.0 mg/L BAP or 0.1 mg/L kinetin was beneficial although some protocols do not require them (Table 4.1).

4.2.1.2 Ovary/Ovule Culture

This technique involves removal of the ovaries 7 DAP and then placing them on medium. Several medium compositions have been evaluated for *B. rapa* × *B. oleracea* crosses, with ½-strength MS without growth regulators supporting higher embryo production rates (Table 4.1). The mean frequency of hybrids obtained per ovary over five crosses was 26 % compared to no seed production through conventional field pollination (Wen et al. 2008). Other studies have shown that ovary culture 9 or 12 DAP was superior, depending on the *B. rapa* and *B. oleracea* genotypes involved in the cross (Zhang et al. 2004). These authors also observed that the highest rate of seeds per ovary depended on the medium composition and cross when using MS or B5 with different concentrations of BA or NAA (Table 4.1). The rate of seed production per ovary reached 72 %.

Table 4.1 Overview of methods used for embryo rescue of oil crops (*Brassica* and sunflowers)

Species/cross	Culture method	Embryo stage (DAP)	Medium (mg/L)	Sucrose (g/L)	Auxin (mg/L)	Cytokinin (mg/L)	Others (mg/L)	Light/dark and temperature	Embryo germination (mg/L)	Seedling or shoot induction (mg/L)	Rooting (mg/L)	Reference
Brassica												
B. carinata × *B. rapa*, *B. carinata* × *B. oleracea*	Embryo	18–22 torpedo to mature	MS, 6 g/L agar	30	0.1 NAA	0.1 Kin		24 h 24 °C	Same	MS, 2.0 BAP, 20 g/L sucrose, 6 g/L agar	Modified KM	Rahman (2004)
B. campestris ssp. pekinensis × *B. oleracea var. acephala*	Embryo	9–11 globular	MS	30	0.1 NAA	2.0 BAP						Zhu and Wei (2009)
B. juncea × *B. napus*, *B. napus* × *B. juncea*	Embryo	10–15 globular	MS, 7.5 g/L agar	30	0.3 NAA	1.5 or 2.0 BAP		16/8 h 22 °C	Same	Same	Same	Zhang et al. (2003)
B. oleracea × *B. carinata*, *B. oleracea* × *B. rapa*	Embryo	24–28 heart to torpedo	MS, 6 g/L agar	30	0.1 NAA	0.1 Kin		24 h 24 °C	Same	MS, 2.0 BAP, 20 g/L sucrose, 6 g/L agar		Rahman (2004)
B. oleracea × *B rapa*	Embryo	16–18 globular to heart	½ MS, 8 g/L agar	30		0.2 BAP	100 colchicine	16/8 h 25 °C	MS, 0.2 BAP	MS, 1.5 BAP, 0.25 NAA	MS, 0.1 NAA	Wen et al. (2008)
B. rapa × *B. oleracea*	Ovaries	4–7	½ MS, 8 g/L agar	30				16/8 h 25 °C	MS, 0.2 BAP	MS,1.5 BAP, 0.25 NAA	MS, 0.1 NAA	Wen et al. (2008)
B. rapa × *B. oleracea*	Ovaries	9–12	MS or B5, pH 5.8, 7.5 g/L agar	30	0.1 or 0.2 NAA	3.0 BAP	500 AC	16/8 h 22 °C	MS, 1.0 BAP, 0.05 NAA	MS, 1.0 BAP, 0.05 NAA	MS, 1.0 BAP, 0.1 NAA	Zhang et al. (2004)
B. juncea × *B. oleracea*, *B. oleracea* × *B. juncea*	Ovules	5–10	MS, 8 g/L agar	30				16/8 h		Momotaz et al. 1998		Tonguç and Griffiths (2004a)

B. napus × *B. oleracea*	Ovules	8	NN				300 CH, 200 Glu	25 °C shaker	B5, 0.1 GA_3			Ripley and Beversdorf (2003)
B. fruticulosa × *B. rapa*	Ovules	2–3	MS, 8 g/L agar	30			500 CH	16/8 h 25 °C	Same	MS, 5 BAP	$^1/_2$ MS	Chandra et al. (2004)
B. carinata × *B. oleracea*	Ovules	5–7	MS, 8 g/L agar	50			500 CH	24 h	MS, 30 g/L sucrose, 8 g/L agar, 16 h light	MS, 30 g/L sucrose, 8 g/L agar, 16 h light	MS, 30 g/L sucrose, 8 g/L agar, 16 h light	Tonguç and Griffiths (2004b)
B. oleracea × *Moricandia arvensis and reciprocal*	Placenta/ embryo	14	MS, 11 g/L agar	30			500 CH		White's, 500 CH, 30 g/L sucrose, 8 g/L agar	15 °C, 16 h light		Bang et al. (1996, 2007)
B. rapa × *B. juncea*	Siliques	9	MS		0.1 NAA	1.0 KIN	1.0 GA_3		MS or MSE (0.1 NAA, 1.0 KIN, 1.0 GA_3)	MS, 1.0 IBA		Srivastava et al. (2004)
Helianthus												
H. annuus × *H. ssp.*	Immature seed	2–4	MS or B5 or B5S or NN, 10 Thia, 1.0 Pyr, 1.0 Nic, 100 myoinositol, 8 g/L agar	60, 90, 120	1.0 NAA		only B5	19/5 h 26 °C	After 1 week in ovulo, embryos were excised and put on fresh medium			Espinasse et al. (1991)

(continued)

Table 4.1 (continued)

Species/cross	Culture method	Embryo stage (DAP)	Medium (mg/L)	Sucrose (g/L)	Auxin (mg/L)	Cytokinin (mg/L)	Others (mg/L)	Light/dark and temperature	Embryo germination (mg/L)	Seedling or shoot induction (mg/L)	Rooting (mg/L)	Reference
H. annuus × *H. ssp*	Embryo	5–10	B5, pH 5.54–5.6, 100 myoinositol, 7 g/L agar	90			B5	24 hLight 25 °C	Floating embryo culture (>1.5 mm); MS, 0.4 Thia, 100 myoinositol, 10 g/L sucrose, 9 g/L agar	After 1–2 weeks shoots transferred to MS		Chandler and Beard (1983); Krauter et al. (1991)
H. annuus × (*H. giganteus, H. laevigatus, H. resinosus, H. pauciflorus, H. decapetalus)*	Embryo	5	B5 pH 5.5, 1.0 Nic, 10 Thia, 1.0 Pyr, 4 g/L myoinositol, 7 g/L agar	120	0.05 NAA		1000 ala, 800 Glu, 160 Ser, 50 Tryp, 10 Cys, MES			B5, 20 g/L sucrose, 7 g/L agar, MES in test tubes		Sukno et al. (1999)
H. annuus × *H. anomalus;* × *H. mollis;* × *H. orgyalis*	Embryo	7	MS, 1.0 Thia, 1.0 Pyr, 0.01 biotin, 1.0 Nic, 8 g/L agar	30	0.02 NAA	0.01 BAP	1.0 Gly	16/8 h 22 °C				Faure et al. (2002)
H. annuus × *H. argophyllus*	Embryo	10	MS	30			MS					Sauca and Lazar (2011)

4.2.1.3 Pistil/Ovary–Ovule Method

Pistils were collected 5–7 DAP and sterilized. They were then cultured on hormone-free MS medium with 50 g/L sucrose, 500 mg/L casein hydrolysate, and 8 g/L agar for 4–7 days (Table 4.1). The enlarged ovules were transferred to MS medium with 30 g/L sucrose, but casein hydrolysate was omitted (Tonguç and Griffiths 2004a). Fifteen hybrid plants were obtained from *B. juncea* by *B. oleracea* crosses using this protocol. This approach has also been used for developing hybrids between *B. carinata* and *B. oleracea* (Tonguç and Griffiths 2004b). However, no hybrid embryos were obtained in the reciprocal cross when using *B. oleracea* as the maternal parent. In crosses of *B. fruticulosa* by *B. rapa*, pistils were excised 2–3 DAP (Chandra et al. 2004). These were cultured on MS medium as above with 30 g/L sucrose, 500 mg/L casein hydrolysate, and 8 g/L agar. After 10–12 days, the enlarged ovules were dissected out of the ovary. From this study, the authors generated 13 seeds from 159 cultured ovaries; 3 of those 13 seeds germinated to produce hybrid plants.

4.2.1.4 Placenta/Ovule–Embryo Culture

Placenta/ovule–embryo culture involves excising the ovules and the attached placenta from the ovary 14 DAP followed by culture on MS medium with 500 mg/L of casein hydrolysate, 30 g/L sucrose, and 11 g/L agar (Bang et al. 1996, 2007; Table 4.1). Embryos were subsequently excised and grown on White's medium with 500 mg/L casein hydrolysate, 30 g/L sucrose, and 8 g/L agar at 15 °C using a 16-h photoperiod (Bang et al. 1996). The placenta/ovule–embryo culture method has been used to develop intergeneric hybrids between *B. oleracea* and *Moricandia arvensis* (Bang et al. 2007). A higher frequency of hybrid production was obtained when *B. oleracea* was used as female parent.

4.2.1.5 Siliques

Developing siliques that are 9 days old were excised, sterilized, and cultured on MS medium with 0.1 mg/L NAA, 1.0 mg/L kinetin, and 1.0 mg/L GA_3 (Srivastava et al. 2004). These cultures were incubated for 30 days, then the siliques were dissected, and the interspecific embryos were excised and cultured on the same medium without growth regulators (Table 4.1). Plants were regenerated, and hybrids were identified by RFLP analysis and cytological studies. Colchicine treatment was applied to the plantlets to double their chromosome number. In the case of crosses between *B. rapa* and *B. nigra* to resynthesize *B. juncea*, the success rate (number of seedlings obtained/number of siliques cultured) ranged from 0 to 5.99 depending on the parental genotypes (Srivastava et al. 2004).

4.2.2 *Helianthus annuus* L. (Sunflower)

Embryo rescue in *Helianthus* species focused on techniques to rescue wide crosses between wild or perennial species and *H. annuus*. Immature embryos were the main explant used, although immature seeds were also useful (Table 4.1). For very young (2–7 DAP) embryos, the two-step method originally developed by Chandler and Beard (1983) continues to be routinely used. Embryos were cultured on a nutrient-rich medium followed by a less rich medium at pH 5.4–5.6, depending on the size of the embryo (Chandler and Beard 1983; Krauter et al. 1991). Espinasse et al. (1991) adapted this method by including an initial culture *in ovulo*. Recent embryo culture protocols use a simpler medium, although the effectiveness of this modification appears to be species specific (Table 4.1; Sukno et al. 1999; Faure et al. 2002; Sauca and Lazar 2011).

4.3 Cereals

4.3.1 *Avena sativa* L. (Oat)

Oat haploids and hybrids have been developed through the use of wide crosses and embryo rescue. Embryo rescue protocols also focused around the development of monosomic addition lines, where maize chromosomes were maintained in a haploid oat background (Rines et al. 2009). The medium developed by Kynast and Riera-Lizarazu (2011) was more effective than previous protocols. The application of auxin and/or gibberellins to the inflorescences at 1–2 DAP was necessary for embryo development (Table 4.2). Recently, the application of a cold treatment immediately after embryo culture was shown to be beneficial for production of haploid embryos (Sidhu et al. 2006).

4.3.2 *Hordeum vulgare* L. (Barley)

Interspecific crosses of barley with *Hordeum bulbosum* and *Elymus canadensis* have been attempted for the purpose of gene transfer and/or haploid production (Dahleen and Joppa 1992; Xu and Kasha 1992). B5 medium was commonly used (Table 4.2). Embryo rescue was successful from crosses of barley with wild rye (Table 4.2); however, the interspecific cross required an initial callus stage before subsequent production of plants (Dahleen and Joppa 1992).

Table 4.2 Overview of methods used for embryo rescue of cereal crops (oats, barley, rice, triticale, sorghum, and corn)

Species/cross	Culture method	Embryo stage (DAP)	Medium (mg/L)	Sucrose (g/L)	Auxin (mg/L)	Cytokinin (mg/L)	Others (mg/L)	Light/dark and temperature	Embryo germination (mg/L)	Seedling or shoot induction (mg/L)	Rooting (mg/L)	Reference
Avena												
A. sativa × *Zea mays*	Embryo	15	½ MS, KR, 2 g/L Gelrite	20			KR	24-h dark 20 °C	14 days	10/14 h		Kynast and Riera-Lizarazu (2011)
Hordeum												
H. vulgare × *Elymus canadensis*	Embryo	~14	B5, 2 g/L gellum gum	12			85 GA_3	24-h light 25 °C				Dahleen and Joppa (1992)
H. bulbosum × *H. vulgare*	Embryo	8–10	B5, 7 g/L agar	20								Xu and Kasha (1992)
Oryza												
O. sativa × *O. australiensis*	Embryo	10–14	¼ MS	7.5			¼ MS	24-h dark 25 °C	Moved to light	3 leaf stage = liquid nutrient solution for 10 days and then to soil		Multani *et al.* (1994)
O. sativa and O. minuta	Embryo	10–14	¼ MS	7.5			¼ MS	24-h dark 28 °C	Moved to light	Liquid nutrient solution and then soil		Mariam et al. (1996)
O. sativa × *(O. minuta;* × *O. officinalis)*	Embryo	11–14	MS, 8 g/L agar	30		1.0 BAP	1,000 CH	25 °C		3 g/L AC		Rodrangboon *et al.* (2002)
Triticale												

(continued)

Table 4.2 (continued)

Species/cross	Culture method	Embryo stage (DAP)	Medium (mg/L)	Sucrose (g/L)	Auxin (mg/L)	Cytokinin (mg/L)	Others (mg/L)	Light/dark and temperature	Embryo germination (mg/L)	Seedling or shoot induction (mg/L)	Rooting (mg/L)	Reference
Wheat × rye	Embryo	12–14	TL, 10 g/L agar	51			2,500 CH	Dark 20 °C	MS, dark, 25 °C	MS, light, 25 °C		Taira and Larter (1978)
Triticale and *Triticale* × *Z. mays* and other *Gramineae*	Embryo	18	MS, 8 g/L agar	30		0.5 Kin	150Glu, 20Arg, 20 Cys, 20 Leu					Pratap et al. (2005)
Sorghum												
S. bicolor × *S. macrospermum*	Embryo	15–20	MS, 7 g/L agar	50			10 Gly, 10 Arg, 10 Tyr, 100 myo- inositol	10/8 h 24 °C		Once embryos had roots and 2–3 leaves, moved to soil		Kuhlman et al. (2010)
S. bicolor × *S. macrospermum*	Embryo	15	MS,7 g/L agar	50			MS	16/8 h 24 °C				Price et al. (2005)
Zea												
Z. mays × *Tripsacum dactyloides*	Embryo	12–14	N6, 0.025 $NaMoO_4$, 0.025 $CuSO_4$, 0.025 $CoCl_2$, HS vitamins	50	0–884 2,4-D	0–292.8 BA	1500 Asp	16/8 h 28–30 °C				Garcia and Molina (1999)
Z. mays ssp. mays × *Z. mays ssp. Parviglumis*	Embryo	21, 23, 30	G	50	0.44–0.22 2,4-D	0.1 KIN	G	24 h dark for 4 weeks 28–30 °C		16/8 h	No hor-mones	Garcia and Molina (2001)

4.3.3 Oryza sp. (Rice)

Rice embryo rescue is required for interspecific hybridization, rescue from degraded endosperm, or rapid generation cycling. Embryo rescue was used to shorten generation time indicating that the technique has become routine (Ohnishi et al. 2011). The most recent protocol deviated from earlier work (Multani et al. 1994; Mariam et al. 1996) in the form of an increase in the MS concentration from ¼ to full strength (Table 4.2; Rodrangboon et al. 2002).

4.3.4 Triticale (X Triticosecale)

The culture of immature triticale embryos was originally for the rescue of tetraploid wheat × diploid rye crosses, or the production of triticale, and the protocol produced by Taira and Larter (1978) appears to have been the basis of culture systems since then (Table 4.2). The latest developments were for the production of haploid triticale where triticale and triticale × wheat crosses were pollinated with *Zea mays* and various other *Gramineae* genera (Pratap et al. 2005). Although plants were regenerated from wide crosses, haploid status was not confirmed.

4.3.5 Sorghum L. and Zea mays L. (Corn)

In maize and sorghum, embryo rescue is employed for the production of interspecific hybrids, polyploidization of lines, revival of germplasm stored for a long time, or rescue of mutant embryos with nonfunctional endosperms used for seed development studies (Garcia and Molina 1995; Consonni et al. 2003; Gutierrez-Marcos et al. 2007). Sorghum embryos (15–20 DAP) rapidly regenerated to plants on MS medium with little supplementation (Table 4.2; Kuhlman et al. 2010; Price et al. 2005). The corn protocol was more complicated, and 12–30-day-old embryos were grown on various media with the addition of 0–884 mg/L 2,4-D and 0–292.8 mg/L BA (Table 4.2; Garcia and Molina 1999; 2001).

4.4 Legumes

4.4.1 Arachis hypogaea L. (Peanut)

Peg tip culture followed by ovule culture and then embryo removal has led to the recovery of hybrids between peanut and its wild relatives (Feng et al. 1996). Growth regulators during peg tip culture were detrimental for embryo development

indicating that the explant provided the necessary hormones required for embryo growth (Table 4.3). The authors related the requirement for embryo rescue to differences in timing of nutrient availability and utilization as well as cytoplasmic connections between the different species as observed by Pattee and Stalker (1991) and Pattee and Mohapatra (1987).

4.4.2 *Cajanus cajan* L. (Pigeonpea)

Crossing the wild pigeonpea with the cultivated species requires a multiple step process from the initial ovule and then embryo culture phases on liquid medium using filter-paper bridges to a solid medium for shoot growth and root induction with a $^{1}/_{10}$-strength MS medium (Mallikarjuna and Moss 1995). Growth regulator content varied from 0.5 mg/L BAP and NAA during ovule culture to an increased concentration (1 mg/L BAP and 0.1 mg/L NAA) during embryo culture and a shift to auxin (0.2 mg/L NAA and 0.1 mg/L IBA) during root induction followed by growth without any growth regulators (Table 4.3). In a subsequent study, Mallikarjuna (1998) increased sucrose concentration to 50 g/L and added 0.5 mg/L each of IAA and kinetin to improve embryo recovery.

4.4.3 *Cicer arietinum* L. (Chickpea)

Mallikarjuna and Muehlbauer (2011) reported hybrids from crosses of *C. arietinum* with *C. pinnatifidum* using a modified liquid MS (ML-6) medium with ovules sitting on filter-paper bridges (Table 4.3). Clarke et al. (2011) also succeeded with the more distantly related species *C. judaicum* using the same medium and filter-paper bridges but increasing the sucrose concentration to 90 g/L. Unfortunately, the hybrids often had poorly formed chloroplasts resulting in albino plantlets, but this trait was genotype specific and was influenced by nuclear factors (Clarke et al. 2011; Kumari et al. 2011). Differences in embryo growth rates between the various species also contribute to the requirement of embryo rescue (Ahmad and Slinkard 1991).

4.4.4 *Glycine max* L. Merr. (Soybean)

Rescue of soybean hybrid embryos requires a complex B5 medium supplemented with organic and amino acids ("B5 long"; Hu et al. 1996; Yeung and Sussex 1979). Depending on their size, embryos were cultured in this liquid medium for 22–61 days and then transferred to a solid maturation and dormancy B5 long medium, with increased sucrose concentration (10 g/L) and activated charcoal (Table 4.3;

Table 4.3 Overview of methods used for embryo rescue of legume species (peanut, pigeonpea, chickpea, soybean, lentil, lupin, medick, bean, clover, and cowpea)

Species/cross	Culture method	Embryo stage (DAP)	Medium (mg/L)	Sucrose (g/L)	Auxin (mg/L)	Cytokinin (mg/L)	Others (mg/L)	Light/dark and temperature	Embryo germination (mg/L)	Seedling or shoot induction (mg/L)	Rooting (mg/L)	Reference
Arachis												
A. hypogaea × *A. glandulifera* *A. duranensis* *A. batizocoi* *A. valida*	Peg, ovule, embryo	10	MS or B5, 6.0 g/L Difco	60			300 CH	Pegs 90 days dark, ovules 30 days dark, embryos light 27 °C	<7 mm: MS, 0.5 BAP, 0.5 GA_3, >7 mm: MS 1.0 BAP, 2.0 GA_3		MS, 2.0 IBA	Feng *et al.* (1996)
Cajanus												
C. platycarpus × *C. cajan*	Ovule, embryo	20 heart to cotyledon	MS and MS pH 5.65 and filter-paper bridges	50	0.5 NAA or 0.5 IAA	0.5 BAP or 0.5 KIN		18/6 h, 26 °C	MS, 0.1 NAA, 1.0 BAP	$^1/_{10}$ MS, 30 g/L sucrose, 7 g/L agar, 0.2 NAA, 0.1 IBA	$^1/_{10}$ MS, 30 g/L sucrose, 7 g/L agar	Mallikarjuna and Moss (1995); Mallikarjuna (1998)
Cicer												
C. arietinum or C. reticulatum × *C. bijugum or C. pinnatifidum or C. judaicum*	Ovule	12–16 heart to cotyledon	MS or ML-6 and MS and filter-paper bridges	30	0.25 IAA	1.0 Zea		16/8 h		MS or ML-6, 30 g/L sucrose, 0.5 IBA, 2 IAA,6.8 g/L agar pH 5.8	MS, 0.5 IBA, 30 g/L sucrose, 7 g/L agar, pH 5.9	Mallikarjuna and Muehlbauer (2011)
Glycine												
G. max × *G. tomentella*	Embryo	20–30	B5 and B5 long	40	0.02 NAA	0.23 BAP	Yeung's, 500 Glu, 100 Ser, 100 Asp, 250 CH	16/8 h, 25 °C		B5 long, 100 g/L sucrose, 5 g/L AC, 10 g/L agar	SH, 10 g/L sucrose, 10 g/L agar	Bodanese-Zanettini *et al.* (1996)
Lens												

(continued)

Table 4.3 (continued)

Species/cross	Culture method	Embryo stage (DAP)	Medium (mg/L)	Sucrose (g/L)	Auxin (mg/L)	Cytokinin (mg/L)	Others (mg/L)	Light/dark and temperature	Embryo germination (mg/L)	Seedling or shoot induction (mg/L)	Rooting (mg/L)	Reference
Lens culinaris × *L. ervoides*	Ovule	14 heart to cotyledon	Modified MS, 0.5 NAA, 0.3 Pyr, 0.1 Thia, 9 g/L agar	100	0.2 IAA	0.5 Zea	0.5 GA_3	72-h dark then 16/8 h, 23–25 °C	Same but 0.2 Zea, 30 g/L sucrose, no GA_3			Cohen et al. (1984)
Lens culinaris × *L. ervoides or L. lamottei or L. nigricans or L. tormentosus*	Ovule	14–18 cotyledon	Modified MS, pH 5.7 8 g/L Pronadisa	10	0.18 IAA	0.17 KIN		12/8 h, 25 °C	Same			Fratini and Ruiz (2011)
Lupinus												
L. angustifolius × *L. luteus*	Embryo	28–35 heart to cotyledon	B5 solid, 7 g/L agar plus 1.5× B5 liquid overlay	30	1.5 IBA		1.5 IBA, 0.53 GA_3, 15 Glu, 725 CH	168-h dark then 168 h 12/12 h, then 16/8 h, 24 °C		¼ B5 in Perlite		Kasten *et al.* (1991)
L. mutabilis × *L. arizonicus L. hartwegii L. mexicanus*	Embryo	28–35 heart to cotyledon	B5, 6 g/L agar	30				168-h dark, then 12/12 h, 24 °C		Same but 20 g/L sucrose		Clements et al. (2008)
Medicago												
M. sativa × *M. rupestris*	Ovule	14–20 torpedo	L2, 1,000 NH_4NO_3, 8 g/L	25				24-h low light, 24.5 °C				McCoy (1985); McCoy and Smith (1986)
Phaseolus												

P. vulgaris × *P. coccineus or P polyanthus*	Pod, ovule, embryo	2–4 pro-embryo	Mod. Phillips et al. (1982), P_o0–P_o1, 5 g/L Difco	143-80	0.1 NAA	10 Adenine	0.095 ABA, 0.25 Glu 0.25 CH 5.5 Tryp	11.5/12.5 h, 20–24 °C	Hu and Zanettini (1995)		Modified Mergeai et al. (1997) 30 g/L sucrose, 0.1 Glu, 0.1 CH, 0.18 GA_3, 5 g/L Difco agar	Geerts et al. (2011)
Trifolium												
T. alexandrium × *T. resupinatum*	Ovule	10–12 heart	MS	30		0.5 KIN		48-h dark then 16/8-h, 22 °C		L2, 0.8 NAA, 0.15 BAP	RL, 0.21 IAA, 25 g/L sucrose	Kaushal et al. (2005)
T. pratense × *T. alpestre*	Embryo	13–19 heart	L2	125	0.06 Pic	2.0 Adenine			L2, 1.0 µg Pic, 15 BAP	L2, 0.5 µg Pic, 0.5 BAP	CR2 + 30 µM coumarin	Phillips et al. (1992)
T. alexandrium × *T. constantino-politanum*	Embryo	heart	MS	30		0.5 KIN		48-h dark, then 16/8-h, 25 °C		MS or ML-6, 0.5 IAA, 2.0 KIN, 3 g/L sucrose, 6.8 g/L agar	RL, 0.8 µg NAA, 0.15 BAP, 25 g/L sucrose	Roy et al. (2004)
Various *Trifolium* crosses	Embryo	14–16 heart	Modified MS, 7 g/L agar	30 Glucose		0.5 KIN		48-h dark, then 16/8-h, 25 °C		LSP3, 0.8 NAA, 0.15 BAP, 25 g/L sucrose	RL, 0.2 IAA, 25 g/L sucrose	Roy et al. (2011)
Trifolium ambiguum × *T occidentale*	Embryo	8 heart	Modified MS, B5, 8 g/L Difco	30	0.05 NAA	1.0 BAP		16/8-h, 26 °C		0.5 BAP, 0.05 NAA		Williams et al. (2011)
Vigna												
V. lanceolata × *V. radiata* *V. luteola* × *V. marina*	Embryo	6–15	MS, 8 g/L Sigma agar	30			500 CH					Palmer et al. (2002)

Bodanese-Zanettini et al. 1996). This is in contrast to many other rescue protocols where the initial medium has high osmotic strength and subsequent media have lower sucrose concentrations. A solid Schenk and Hildebrandt (1972)-based medium with 10 g/L sucrose was used for embryo germination and seedling development. Only hybrids with *G. tomentalla* collected 20–30 DAP could be recovered; other crosses could not be rescued since no medium has been developed for embryos younger than 8 DAP.

4.4.5 Lens culinaris Medik. (Lentil)

Two methods of embryo rescue were successfully used for the recovery of hybrids between the cultivated lentil and some of its wild relatives, but success seemed to be genotype specific. Cohen et al. (1984) used an initial high osmotic culture medium supplemented with IAA, zeatin, and gibberellin for rescue of 14-day-old hybrid seeds (Table 4.3). After 2 weeks of culture, embryos were excised and transferred to a medium with reduced sucrose content (30 g/L) and only zeatin added. In contrast, Fratini and Ruiz (2011) used a low osmotic medium (10 g/L sucrose) for the initial 18-day-old seed culture and replaced zeatin with kinetin. Embryos were also excised after 2 weeks and cultured on the same medium using a short-day setting to improve vegetative growth. However, root development of developing hybrids is often poor. Grafting of hybrid shoots onto faba bean rootstock can be used to overcome poor rooting and has been successfully used for developing and multiplying hybrid material (Gurusamy et al. 2012; S. Saha, personal communication; Yuan et al. 2011).

4.4.6 Lupinus sp. (Lupin)

Crosses between the narrow-leaf (*L. angustifolius*; 2n=40) and yellow (*L. luteus*; 2n=52) lupin are complicated due to different chromosome numbers. Kasten et al. (1991) obtained a single hybrid which eventually died due to chlorophyll deficiencies. They used a B5 medium with a liquid overlay of 1.5 times the strength of B5 medium (Table 4.3). Plantlets were subsequently transferred to sterile Perlite™ with ¼-strength B5 medium. Clements et al. (2008) reported obtaining hybrids from lupin crosses involving *L. mutabilis* with *L. arizonicus*, *L. hartwegii*, and *L. mexicanus*. A simple B5 medium with 30 g/L sucrose was used, and embryos were subcultured on the same medium but with 20 g/L sucrose.

4.4.7 Medicago sativa L. (Alfalfa)

McCoy (1985) and McCoy and Smith (1986) developed an ovule–embryo rescue protocol for the development of interspecific *Medicago* hybrids. Ovules were cultured on L2 (Phillips and Collins 1979) medium with 1 g/L ammonium nitrate

(Table 4.3). Interestingly, ovule culture was time limited to 6–9 days. Shorter or longer culture was detrimental, and embryos needed to be excised and transferred to the same medium for further development.

4.4.8 *Phaseolus vulgaris* L. (Dry Bean)

The bean embryo rescue protocol developed by Geerts et al. (2011) is unusual since the authors succeeded in rescuing 2–5-day-old hybrids using a six-step procedure consisting of (1) pod culture for 5–10 days, (2) embryo excision and culture followed by (3) embryo maturation and dehydration for 14 days, (4) embryo germination, (5) rooting of shoots, and (6) hardening of plantlets (Table 4.3). The authors noted that *P. vulgaris* × *P. polyanthus* crosses aborted due to a lack of division of the primary endosperm nuclei, thus limiting nutrient exchange between embryo and endosperm. Embryo starvation was overcome by dripping a low-nutrient solution (P_oO; modified Phillips et al. 1982) with a high sucrose (143 g/L) concentration onto the young pods. Low concentrations of ABA and NAA were also added. The sucrose concentration was then gradually lowered to 102 and 80 g/L (P_o1) over a 5–7-day period. After excision, embryos were cultured on P_o1 medium for 2 weeks but with 1 mg/L BAP and then exposed to dehydration using a G6 (modified Hu and Zanettini 1995) medium with 100 g/L sucrose, activated charcoal, and lower nutrient concentration except for 2.5 g/L KNO_3. Embryo germination, shoot development, and rooting were accomplished on G7 (modified Mergeai et al. 1997) medium with a reduced sucrose concentration (30 g/L) and a combination of BAP and GA_3.

4.4.9 *Trifolium* sp. (Clover)

Several research groups have developed embryo rescue protocols for clover (Table 4.3; Kaushal et al. 2005; Phillips et al. 1992; Roy et al. 2004, 2011; Williams et al. 2011). The most recent techniques are summarized by Roy et al. (2011). Depending on the crosses, heart-shaped embryos were excised and transferred to a variety of media (Table 4.3). Interestingly, 15 % sterile cucumber juice was added; this juice contains among other nutrients potassium, magnesium, and sulfur as well as vitamins, amino acids, and silica according to Nakajima et al. (1969).

4.4.10 *Vigna* sp.

The genus *Vigna* contains a variety of crop species, and Palmer et al. (2002) obtained hybrids from crosses between a native Australian species, *V. lanceolata*, and the wild mungbean (*V. radiata* ssp. sublobata) by culturing 9–15-day-old immature

embryos on MS medium with 30 g/L sucrose, 500 mg/L casein hydrolysate, and 8 g/L agar (Table 4.3). Addition of growth regulators was detrimental to germination of embryos.

4.5 Horticultural Species

4.5.1 *Allium (Onion)*

The genus *Allium* contains hundreds of distinct species, which includes many important vegetables like onion (*Allium cepa*), garlic (*A. sativum*), leek (*A. ampeloprasum*), and chive (*A. schoenoprasum*). In an attempt to cross Welsh onion (*A. fistulosum*) with Japanese garlic (*A. macrostemon* Bunge), ovaries at 7 DAP were separated and cultured on BDS medium (Dunstan and Short 1977). One month after ovary culture, germinated embryos were excised and transferred to BDS medium with 1 mg/L BAP, and rooting was achieved by subculturing shoots onto B5 medium (Table 4.4, Umehara et al. 2006). An interspecific hybrid between leek (*A. ampeloprasum*) and garlic (*A. sativum*) was also produced by using a fertile garlic clone as a pollen donor and ovary culture on LS (Linsmaier and Skoog 1965) medium (Yanagino et al. 2003).

4.5.2 *Capsicum baccatum (Chilli Pepper)*

Hossain et al. (2003) reported that hybrid plants from *C. annuum* and *C. frutescens* were produced only from embryos at 28–33 DAP when cultured on MS medium supplemented with casein hydrolysate, yeast extract, coconut water, and growth regulators (Table 4.4). Yoon et al. (2004) rescued *C. annuum* and *C. baccatum* hybrid embryos (35–40 DAP) from torpedo to early cotyledonary stages and cultured them on MS medium supplemented with 10 µg/L IAA and 10 µg/L GA_3. Intact hybrid plants were regenerated, but few plants could be regenerated from embryos at the heart-shape stage and no plants were obtained from globular embryos (Yoon et al. 2006).

4.5.3 *Citrus Species*

Embryo rescue is important for citrus sexual breeding because polyembryony can interfere with hybrid recovery. Tan et al. (2007) reported that success of embryo culture was closely associated with the developmental stage of the embryo and 80 DAP was the optimal time for rescue of crosses between satsuma mandarin (*Citrus unshiu* Marc), red tangerine (*C. reticulata* Blanco), and trifoliate orange (*Poncirus trifoliata* (L.)

Table 4.4 Overview of methods used for embryo rescue of horticultural species

Species/crosses	Culture method	Embryo stage (DAP)	Medium (mg/L)	Sucrose (g/L)	Auxin (mg/L)	Cyto-kinin (mg/L)	Others (mg/L)	Light/dark and Temperature	Embryo germination (mg/L)	Seedling or shoot induction (mg/L)	Rooting (mg/L)	Reference
Allium												
A. fistulosum × *A. macrostemon*	Ovary	7	BDS, 2 g/L gellan gum	30				16 h/8 h, 25 °C		BDS, 15 g/L sucrose, 2 g/L gellan gum, 1.0 BAP	BDS, 15 g/L sucrose, 2 g/L Gelan gum	Umehara et al. (2006)
A. ampeloprasum × *A. sativum*	Ovary	4–6	LS	50				14 h/10 h, 20 °C		MS, 30 g/L sucrose		Yanagino et al. (2003)
Capsicum												
C. annuum L. × *C. frutescens L.*	Embryo	28–33	MS		0.05 NAA		0.5 GA_3 500 CH, 500 YE, 150 mL/L CW			MS, 20 ascorbic acid		Hossain et al. (2003)
C. annuum × *C. baccatum*	Embryo	35–40 torpedo to early cotyle-don	MS	80	0.01 IAA		0.01 GA_3	16 h/8 h, 25 °C		MS, 30 g/L sucrose		Yoon et al. (2006)
Citrus												
C. unshiu × × *Poncirus trifoliata, C. unshiu* × *C. reticulate*	Embryo	80	MT, pH 5.8, 8 g/L agar	40			0.5–1.0 GA_3	16-h/8 h, 26 °C		MT, 0.5 BAP, 0.5 KIN, 0.1 NAA, 25 g/L sucrose	RMAN medium	Tan et al. (2007)
Cucumis												
C. hystrix × *C. sativus* L.	Embryo	16 early heart	MS, pH 6, 8–12 g/L agar	30				16 h/8 h, 25 °C				Chen et al. (2003)
C. anguria × *C. zeyheri*	Embryo	14–42	MS, 8 g/L agar	20	0.01 IBA	0.01 BAP	5 % CW	Dark, 22–24 °C for 6 weeks then 22 °C, 16-h day/18 °C 8-h night				Skalova et al. (2008a)

(continued)

Table 4.4 (continued)

Species/crosses	Culture method	Embryo stage (DAP)	Medium (mg/L)	Sucrose (g/L)	Auxin (mg/L)	Cyto-kinin (mg/L)	Others (mg/L)	Light/dark and Temperature	Embryo germination (mg/L)	Seedling or shoot induction (mg/L)	Rooting (mg/L)	Reference
Lycopersicon												
L. esculentum × *L. chilense, L. peruvianum, L. hirsutum*	Embryo, immature seed	25 globular	HLH			2.25 BAP	1 YE	Dark 36 h then 16 h/8 h, 24–26 °C		MS, 30 g/L sucrose, 4.0 IAA, 4.0 KIN, 1.0 Zea	MS, 10 g/L sucrose, 0.1 IAA	Pico et al. (2002)
Prunus												
P. persica × *P. armeniac, P. salicina*	Embryo	70	½ MS, 6.5 g/L agar	35	0.5 IBA	4.0 BAP		12 h/12 h, 25 °C		MS, 1.0 IBA, 1.0 BAP	MS, 0.5 IAA, 0.2 NAA	Liu et al. (2007)
P. salicina Lindl. × *Armeniaca vulgaris* Lam.	Embryo	42–56	MS, pH 5.8, 6.5 g/L agar	35	0.3 IAA	2.0 BAP		12 h/12 h, 25 °C		MS, 0.3 IAA, 1.5 BAP	½ MS, 0.8 IAA	Yang et al. (2004)
Solanum												
S. tuberosum × *S. pinnatisectum*	Embryo	15–20	MS pH 5.6 and 7 g/L agar	40			1,000 CH	16 h/8 h, 18–22 °C				Ramon and Hanneman (2002)
Vitis												
V. vinifera × *V. amurensis*	Ovule	49	ER, pH 5.8, 6 g/L agar	60			260 Asp, 3 g/L AC	14 h/10 h, 25 °C	WPM, 2.25 BAP, 1.5 g/L AC		MS, 1 IBA	Tian et al. (2008); Tian and Wang (2008)

Base media: *B5* (Gamborg et al. 1968), *B5G* (B5 medium with GA_3 added), *B5 long* (Organic supplements according to Hu et al. 1996 and amino acids according to Yeung and Sussex 1979), *B5S* (B5 medium without vitamins and amino acids), *BDS* (Dunstan and Short 1977), *Cohen* (Cohen et al. 1984), *CR2* (Cohen et al. 1984), *ER* (Emershad and Ramming 1994), *G* (Garcia et al. 1991), *HLH saline solution* (Neal and Topolesky 1983), *KR* (Kynast and Riera-Lizarazu 2011), *L2* (Phillips and Collins 1979), *LIH* (Phillips et al. 1982), *LS* (Phillips et al. 1982), *LS* (Linsmaier and Skoog 1965), *LSP2* (Phillips et al. 1982), *LSP3* (Phillips and Collins 1984), *Mergeai* (Mergeai *et al*. 1997), *MS* (Murashige and Skoog 1962), *ML-6* (Kumar et al. 1988), *MT* (Murashige and Tucker 1969), *N6* (Chu 1978), *NN* (Nitsch and Nitsch, 1969), *RL* (Phillips and Collins 1984), *RMAN* (Grosser and Gmitter, 1990), *SH* (Schenk and Hildebrandt 1972), *TL* (Taira and Larter 1978), *White* (White 1963), *Yeung's amino acids* (Yeung and Sussex 1979).

Abbreviations: *AC* Activated charcoal, *Ala* Alanine, *Arg* Arginine, *BAP* 6-benzylaminopurine, *CH* Casein hydrolysate *CW* Coconut water, *Cys* Cysteine, *2,4-D* 2,4-Dichlorophenoxyacetic acid, *DAP* Days after pollination, *Fe* FeEDTA, *Glu* Glutamine, *Gly* Glycine, *KIN* Kinetin, *Leu* Leucin, *MES* 2-(N-morpholino)-ethane sulfonic acid, *NAA* α-Naphthalene acetic acid, *Pic* Picloram, *Nic* Nicotinic acid, *Pyr* Pyridoxine, *Ser* Serine, *Thia* Thiamine, *Tryp* Tryptophan, *Tyr* Tyrosine, *YE* Yeast extract, *Zea* Zeatin

Raf.). In crosses of satsuma mandarin with trifoliate orange, MT (Murashige and Tucker 1969) medium with 0.5 mg/L GA_3 resulted in higher embryo germination rates, while 1.0 mg/L GA_3 was required for crosses between red tangerine with trifoliate orange (Tan et al. 2007, Table 4.4).

4.5.4 *Cucumis sativus* L. (Cucumber)

To obtain interspecific hybrids from crosses between *C. hystrix* Chakr. and *C. sativus* L., immature embryos (16 DAP) were dissected out and cultured on MS hormone-free medium until plantlets developed (Table 4.4; Chen et al. 2003). One-week-old embryos from crosses of *C. anguria* × *C. zeyheri* formed mostly callus through intact seed culture, but plants were obtained by culture of 2–6-week-old embryos (Table 4.4; Skalova et al. 2008a). MS medium containing 5 % coconut water was suitable for the initial embryo germination, and a medium with 20 mg/L ascorbic acid was best for embryo development and plant recovery (Skalova et al. 2008a, b).

4.5.5 *Lycopersicon esculentum* Mill. (Tomato)

To overcome barriers in crossing wild with cultivated tomatoes, a combination of two or more methods was necessary such as crossing with pollen mixtures (10:1, wild: cultivated), stigma treatment with 100 mg/L H_3BO_3, spraying pistils with 75 mg/L GA_3, and embryo rescue (Pico et al. 2002). Globular stage embryos (25 DAP) cultured in HLH (Neal and Topolesky 1983) saline solution supplemented with 2.25 mg/L BA and 1 mg/L yeast extract (YE) were suitable for callus induction. Calli were then transferred to MS medium supplemented with different growth regulators to regenerate shoots and induce rooting (Table 4.4, Pico et al. 2002).

4.5.6 *Prunus* Species

To overcome incompatibilities between parents in wide hybridization of *Prunus* species, a three-step strategy was established by Liu et al. (2007). First, pollen was exposed to an electrostatic field (434.78 kV/m) to enhance germination and fruit set; second, immature embryos were not rescued until 10 weeks after pollination; and third, appropriate media were selected for embryo germination and multiplication of the hybrid seedlings (Table 4.4). Attempts to create new germplasm by wide hybridization between plum (*P. salicica*) and apricot (*P. armeniaca*) also found that the proper stage for embryo rescue and optimum culture media were important factors (Table 4.4; Yang et al. 2004). Kukharchyk and Kastrickaya (2006) reported that the timing of embryo rescue varied depending on the cross combination of genotypes used.

4.5.7 *Solanum tuberosum subsp. tuberosum (Potato)*

Ramon and Hanneman (2002) crossed tuber-bearing late blight-resistant wild relatives of the cultivated potato from central Mexico with *Solanum tuberosum* subsp. *tuberosum*. Embryos were dissected out and rescued 15–20 DAP and a haploid cultivar developed. A second pollination combined with embryo rescue resulted in one true *S. tuberosum* subsp. *tuberosum* haploid × *S. pinnatisectum* hybrid, which showed a high degree of late blight resistance in the field, equivalent to the resistance in its *S. pinnatisectum* parent (Table 4.4).

4.5.8 *Vitis vinifera L. (Grape)*

Tian et al. (2008) established an embryo rescue protocol that can be applied to 11 different cross combinations of grape. In their protocol, ovules were cultured 7 weeks after pollination in a double-phase ER medium (Emershad and Ramming 1994) containing 60 g/L sucrose and 3 g/L activated charcoal (Table 4.4). After 8–12 weeks of culture, embryos were dissected out and transferred onto WPM medium (McCown and Lloyd 1981) supplemented with growth regulators until plantlet recovery. Culture duration of in vitro ovules has an important role in plant development since more than 16 weeks of culture reduced the regeneration ability of embryos (Tian et al. 2008). Further optimization of this protocol found that the addition of 0.26 g/L asparagine significantly increased the embryo recovery rate (Tian and Wang 2008).

4.6 Conclusions

Although the role of embryo rescue is similar across species, the methods available are as diverse as the species cultured (Tables 4.1, 4.2, 4.3, and 4.4). Changes in medium components are a major route of adapting protocols to each species with differences in base medium, vitamins, sucrose concentration, and growth regulators added. Growth conditions vary from initial growth in light or darkness to changes in the optimum photoperiod and growth temperatures. Monocot culture tends to be more direct than dicot culture, where many protocols require a multi-step approach from pod to ovule culture to embryo rescue, shoot regeneration, and root induction. The number of explants available in *Brassica*, from embryo, pod/silique, and ovaries to immature seeds (ovules) with attached placentas, allows species-specific protocol adaptations. The parental genotypes and the direction of the cross are also important in the *Brassica* species. Legume species likewise stand out because some researchers managed to obtain hybrids from embryos as young as 2 days after pollination (Geerts et al. 2011). However, many species such as soybean and chickpea still require procedures for rescue of very young embryos. Others have overcome hurdles such as poor rooting in

tissue culture by using grafting onto a different species (Yuan et al. 2011). In all species, the role of the methodology was to rescue aborting embryos and ensure the survival of unique germplasm. These techniques remain a useful component in any breeding program where wide or interspecific crosses are preformed, where rapid cycling through generations is used, and where germplasm preservation is required.

References

Ahmad F, Slinkard AE (1991) Relative growth rates in the annual C*icer* species. Ann Bot 68:489–493

Bang SW, Kaneko Y, Matsuzawa Y (1996) Production of intergeneric hybrids between *Raphanus* and *Moricandia arvensis*. Breed Sci 46:45–51

Bang SW, Sugihara K, Jeung BH, Kaneko R, Satake E, Kaneko Y, Matsuzawa Y (2007) Production and characterization of intergeneric hybrids between *Brassica oleracea* and a wild relative *Moricandia arvensis*. Plant Breed 126:101–103

Bodanese-Zanettini MH, Lauxen MS, Richter SNC, Cavalli-Molina S, Lange CE, Wang PJ, Hu CY (1996) Wide hybridization between Brazilian soybean cultivars and wild perennial relatives. Theor Appl Genet 93:703–709

Chandler JM, Beard BH (1983) Embryo culture of *Helianthus* hybrids. Crop Sci 23:1004–1007

Chandra A, Gupta ML, Banga SS, Banga SK (2004) Production of an interspecific hybrid between *Brassica fruticulosa* and *B. rapa*. Plant Breed 123:497–498

Chen JF, Staub J, Qian C, Jiang J, Luo X, Zhuang F (2003) Reproduction and cytogenetic characterization of interspecific hybrids derived from *Cucumis hystrix* Chakr. × *Cucumis sativus* L. Theor Appl Genet 106:688–695

Chu CC (1978) The N6 medium and its applications to anther culture of cereal crops. In: Proceedings of symposium on plant tissue culture. Beijing, China, Science Press, 43–50

Clarke HJ, Kumari M, Khan TN, Siddique KHM (2011) Poorly formed chloroplasts are barriers to successful interspecific hybridization in chickpea following *in vitro* embryo rescue. Plant Cell Tiss Org Cult 106:465–473

Clements JC, Prilyuk L, Quealy JA, Francis GW (2008) Interspecific crossing among the New World Lupin Species for *Lupinus mutabilis* Crop Improvement. In: Palta JA, Berger JB (eds) 12th international lupin conference. International Lupin Association, Fremantle, Western Australia, pp 14–18

Cohen D, Ladizinsky G, Ziv M, Muehlbauer FJ (1984) Rescue of interspecific *Lens* hybrids by means of embryo culture. Plant Cell Tiss Org Cult 3:343–347

Consonni G, Aspesi C, Barbante A, Dofini S, Giuliani C, Giulini A, Hansen S, Brettschneider R, Pilu R, Gavazzi G (2003) Analysis of four maize mutants arrested in early embryogenesis reveals an irregular pattern of cell division. Sex Plant Reprod 15:281–290

Dahleen LD, Joppa LR (1992) Hybridization and tissue culture of *Hordeum vulgare* × *Elymus canadensis*. Genome 35:1045–1049

Dunstan DI, Short KC (1977) Improved growth of tissue cultures of the onion *Allium cepa*. Physiol Plant 41:70–72

Emershad RL, Ramming DW (1994) Somatic embryogenesis and plant development from immature zygotic embryos of seedless grapes (*Vitis vinifera* L.). Plant Cell Rep 14:6–12

Espinasse A, Volin J, Dybing CD, Lay C (1991) Embryo rescue through *in ovulo* culture in *Helianthus*. Crop Sci 31:102–108

Faure N, Serieys H, Berville A, Cazaux E, Kaan F (2002) Occurrence of partial hybrids in wide crosses between sunflower (*Helianthus annuus*) and perennial species *H. mollis* and *H. orgyalis*. Theor Appl Genet 104:652–660

Feng Q, Stalker HT, Pattee HE (1996) Plant recovery of selfs and interspecific hybrids of *Arachis* by *in vitro* culture of peg tips. Crop Sci 36:1660–1666

Fratini R, Ruiz ML (2011) Wide crossing in lentil through embryo rescue. In: Thorpe TA, Young EC (eds) Plant Embryo Culture: Methods and Protocols. Methods in Molecular Biology, vol 710. Humana press, New York, NY, pp 131–139

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybeanroot cells. Exp Cell Res 50:150–158

Garcia MD, Molina MC (1995) Embryo in-viability in crosses of tetraploid (2n=40) × diploid (2n=20) can be overcome by embryo rescue. Maize Genetics Coop Newsl 69:90–91

Garcia MD, Molina MC (1999) Plant regeneration of maize-*Tripsacum* hybrids from organogenic or embryogenic long-term callus cultures. Maize Genetics Coop Newsl 73:64–65

Garcia MD, Molina MC (2001) Embryo rescue and induction of somatic embryogenesis as a method to overcome seed in-viability in *Zea mays* ssp. *mays* × *Zea mays* ssp. *parviglumis* crosses. Biologia Plant 44:497–501

Garcia MD, Molina MC, Caso O (1991) *In vitro* culture of 0.15–0.25mm immature embryos. I. Picloram effects. Maize Genetics Coop Newsl 65:76–77

Geerts P, Toussaint A, Mergeai G, Baudoin JP (2011) *Phaseolus* immature embryo rescue technology. In: Thorpe TA, Young EC (eds) Plant Embryo Culture: Methods and Protocols. Humana press, New York, NY, pp 117–129

Grosser JW, Gmitter FG Jr (1990) Protoplast fusion and citrus improvement. Plant Breed Rev 8:339–374

Gurusamy V, Warkentin TD, Vandenberg A (2012) Grafting pea, faba bean, and lentil to improve pulse crop breeding. Can J Plant Sci 92:31–38

Gutierrez-Marcos JF, Pra MD, Giulini A, Costa LM, Gavazzi G, Cordelier S, Sellam O, Tatout C, Paul W, Perez P, Dickinson HG, Consonni G (2007) *empty pericarp4* encodes a mitochondrion-targeted pentatricopeptide repeat protein necessary for seed development and plant growth in maize. Plant Cell 19:196–210

Hossain MA, Minami M, Nemoto K (2003) Immature embryo culture and interspecific hybridization between *Capsicum annuum* L. and *C. frutescens* L. viaembryo rescue. Jap J Trop Agric 47:9–16

Hu CY, Yin GC, Bodanese-Zanettini MH (1996) Haploid of soybean. In: Jain SM, Sopory SK, Veilleux RE (eds) *In vitro* Haploid Production in Higher Plants. Kluwer, Dordrecht, pp 377–395

Hu CY, Zanettini MHB (1995) Embryo culture and embryo rescue for wide cross hybrids. In: Gamborg OL, Phillips GC (eds) Plant Cell, Tissue and Organ Culture: fundamental methods. Springer Verlag, Berlin, pp 129–141

Inomata N (1993) Embryo rescue techniques for wide hybridization. In: Labana KS, Banga SS, Banga SK (eds) Breeding oilseed *Brassicas*. SpringerVerlag, Berlin, pp 94–107

Kasten W, Paradies T, Kunert R, Straka P (1991) Progress in realization of interspecific hybrids in the genus *Lupinus* by means of embryo rescue technique. Biol Zentralblatt 110:301–309

Kaushal DR, Malaviya DR, Roy AK, Kumar B, Tiwari A (2005) *Trifolium alexandrinum* × *T. resupinatum*—interspecific hybrids developed through embryo rescue. Plant Cell Tiss Org Cult 83:137–144

Krauter R, Steinmetz A, Friedt W (1991) Efficient interspecific hybridization in the genus *Helianthus* via “embryo rescue” and characterization of the hybrids. Theor Appl Genet 82:521–525

Kuhlman LC, Burson BL, Stelly DM, Klein PE, Klein RR, Price HJ, Rooney WL (2010) Early-generation germplasm introgression from *Sorghum macrospermum* into sorghum (*S. bicolor*). Genome 53:419–429

Kukharchyk N, Kastrickaya M (2006) Embryo rescue techniques in *Prunus* L. Breeding. J Fruit Ornam Plant Res 14(Suppl 1):129–135

Kumar AS, Gamborg OL, Nabors MW (1988) Plant regeneration from cell suspension cultures of *Vigna aconitifolia*. Plant Cell Rep 7:138–141

Kumari M, Clarke HJ, Colas des Francs-Small C, Small I, Khan TN, Siddique KHM (2011) Albinism does not correlate with biparental inheritance of plastid DNA in interspecific hybrids in *Cicer* species. Plant Sci 180:628–633

Kynast RG, Riera-Lizarazu O (2011) Development and use of oat-maize chromosome and radiation hybrids. In: Birchler JS (ed) Plant Chromosome Engineering: Methods and Protocols, Methods in Molecular Biology, vol 701. Humana Press, New York, NY, pp 259–284

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18:100–127

Liu W, Chen XS, Liu GJ, Liang Q, He TM, Feng JR (2007) Interspecific hybridization of *Prunus persica* with *P. armeniaca* and *P. salicina* using embryo rescue. Plant Cell Tiss Org Cult 88:289–299

Mallikarjuna N (1998) Ovule culture to rescue aborting embryos from pigeonpea (*Cajanus cajan* (L.) Millspaugh) wide crosses. Indian J Exp Biol 36:225–228

Mallikarjuna N, Moss JP (1995) Production of hybrids between *Cajanus platycarpus* and *Cajanus cajan*. Euphytica 83:43–46

Mallikarjuna N, Muehlbauer FJ (2011) Chickpea hybridization using *in vitro* techniques. In: Thorpe TA, Young EC (eds) Plant Embryo Culture: Methods and Protocols, Methods in Molecular Biology, vol 710. Humana press, New York, NY, pp 93–105

Mariam AL, Zakri AH, Mahani MC, Normah MN (1996) Interspecific hybridization of cultivated rice, *Oryza sativa* L. with the wild rice, *O. minuta* Presl. Theor Appl Genet 93:664–671

McCown BH, Lloyd G (1981) Woody plant medium, a mineral nutrient formula for micro culture of woody plant species. Hortscience 16:453

McCoy TJ (1985) Interspecific hybridization of *Medicago sativa* L. and *M. rupestris* M.B. using ovule-embryo culture. Can J Genet Cytol 27:238–245

McCoy TJ, Smith LY (1986) Interspecific hybridization of perennial *Medicago* species using ovule-embryo culture. Theor Appl Genet 71:772–783

Mergeai G, Schmit V, Lecomte B, Baudoin JP (1997) Mise au point d'une technique de culture *in vitro* d'embryons immatures de *Phaseolus*. Biotechnol Agron Soc Environ 1:49–58

Momotaz A, Kato M, Kakihara F (1998) Production of intergeneric hybrids between Brassica and Sinapis species by means of embryo rescue technique. Euphytica 103:123–130

Multani DS, Jena KK, Brar DS, de los Reyes BG, Angeles ER, Khush GS (1994) Development of monosomic alien addition lines and introgression of genes from *Oryza australiensis* Domin. to cultivated rice *O. sativa* L. Theor Appl Genet 88:102–109

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Murashige T, Tucker OPH (1969) Growth requirements of citrus tissue culture. Proceedings of the First International Citrus Symposium, vol. 3. p 1155–1161

Nakajima T, Doyama Y, Matsumoto H (1969) *In vitro* culture of excised ovules of white clover *Trifolium repens* L. Jpn J Breed 19:373–378

Neal CA, Topolesky LD (1983) Effects of the basal medium on growth of immature embryos *in vitro*. J Am Soc Hort Sci 108:434–438

Nishi S, Kawata J, Toda M (1959) In the breeding of interspecific hybrids between two genomes "c" and "a" of *Brassica* through the application of embryo culture techniques. Jpn J Breed 5:215–222

Nitsch C, Nitsch JP (1967) The induction of flowering stem segments of *Plumbago indica* L. 1. the production of vegetative buds. Planta 72:355–370

Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163:85–87

Ohnishi T, Yoshino M, Yamakawa H, Kinoshita T (2011) The biotron breeding system: a rapid and reliable procedure for genetic studies and breeding in rice. Plant Cell Physiol 52:1249–1257

Palmer JL, Lawn RJ, Adkins SW (2002) An embryo-rescue protocol for *Vigna* interspecific hybrids. Aust J Bot 50:331–338

Pattee HE, Mohapatra SC (1987) Anatomical changes during ontogeny of the peanut (*Arachis hypogea* L.) fruit: mature megagametophyte through heart-shaped embryo. Bot Gaz 148: 156–164

Pattee HE, Stalker HE (1991) Comparative embryo sac morphology at anthesis of cultivated and wild species of *Arachis*. Ann Bot 68:511–517

Phillips GC, Collins GB (1979) *In vitro* tissue culture of selected legumes and plant regeneration from callus cultures of red clover. Crop Sci 19:59–64

Phillips GC, Collins GB (1984) Red clover and other forage legumes. In: Sharp WR, Evans DA, Ammirato PV, Yamada Y (eds) Handbook of Plant Cell Culture, vol 2. Macmillan, New York, NY, pp 169–210

Phillips GC, Collins GB, Taylor NL (1982) Interspecific hybridization of red clover (*Trifolium pretense* L.) with *T. sarosiense* Hazsl. using *in vitro* embryo rescue. Theor Appl Genet 62:17–24

Phillips GC, Grosser JW, Berger S, Taylor NL, Collins GB (1992) Interspecific hybridization between red clover and *Trifolium alpestre* using *in vitro* embryo rescue. Crop Sci 32: 1113–1115

Pico B, Herraiz J, Ruiz JJ, Nuez F (2002) Widening the genetic basis of virus resistance in tomato. Sci Hort 94:73–89

Pratap A, Sethi GS, Chaudhary HK (2005) Relative efficiency of different *Gramineae* genera for haploid induction in triticale and triticale × wheat hybrids through the chromosome elimination technique. Plant Breed 124:147–153

Price HJ, Hodnett GL, Burson BL, Dillon SL, Rooney WL (2005) A *Sorghum bicolor* × *S. macrospermum* hybrid recovered by embryo rescue and culture. Aus J Bot 53:579–582

Rahman MH (2004) Optimum age of siliques for rescue of hybrid embryos from crosses between *Brassica oleracea, B. rapa* and *B. carinata*. Can J Plant Sci 84:965–969

Ramon M, Hanneman RE Jr (2002) Introgression of resistance to late blight (*Phytophthora infestans*) from *Solanum pinnatisectum* into *S. tuberosum* using embryo rescue and double pollination. Euphytica 127:421–435

Rines HW, Phillips RL, Kynast RG, Okagaki RJ, Galatowitsch MW, Huettl PA, Stec AO, Jacobs MS, Suresh J, Porter HL, Walch MD, Cabral CB (2009) Addition of individual chromosomes of maize inbreds B73 and Mo17 to oat cultivars Starter and Sun II: maize chromosome retention, transmission, and plant phenotype. Theor Appl Genet 119:1255–1264

Ripley VL, Beversdorf WD (2003) Development of self-incompatible *Brassica napus*: (I) introgression of S-alleles from *Brassica oleracea* through interspecific hybridization. Plant Breed 122:1–5

Rodrangboon P, Pongtongkam P, Suputtitada S, Adachi T (2002) Abnormal embryo development and efficient embryo rescue in interspecific hybrids, *Oryza sativa* × *O. minuta* and *O. sativa* × *O. officinalis*. Breed Sci 52:123–129

Roy AK, Malaviya DR, Kaushal P (2011) Generation of interspecific hybrids of *Trifolium* using embryo rescue techniques. In: Thorpe TA, Young EC (eds) Plant Embryo Culture: Methods and Protocols, Methods in Molecular Biology, vol 710. Humana Press, New York, NY, pp 141–151

Roy AK, Malaviya DR, Kaushal P, Kumar B, Tiwari A (2004) Interspecific hybridization of *Trifolium alexandrinum* with *T. constantinopolitanum* using embryo rescue. Plant Cell Rep 22:705–710

Sauca F, Lazar DA (2011) Scientific results regarding the gene(s) introgression of drought-resistance to *Helianthus annuus* species, using embryo rescue. Rom Biotechnol Lett 16:3–8

Schenk RU, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Bot 50:199–204

Sidhu PK, Howes NK, Aung T, Zwer PK, Davies PA (2006) Factors affecting oat haploid production following oat × maize hybridization. Plant Breed 125:243–247

Skalova D, Dziechciarkova M, Lebeda A, Kristkova E, Navratilova B (2008a) Interspecific hybridization of *Cucumis anguria* and *C. zeyheri* via embryorescue. Biologia Plant 52:775–778

Skalova D, Navratilova B, Lebeda A (2008b) Embryo rescue of cucumber (*Cucumis sativus*), muskmelon (*C. melo*) and some wild *Cucumis species* (*C. anguria*, *C. zeyheri*, and *C.metuliferus*). J Appl Bot Food Qual 82:83–89

Srivastava A, Mukhopadhyay A, Arumugam N, Gupta V, Verma JK, Pental D, Pradhan AK (2004) Re-synthesis of *Brassica juncea* through interspecific crosses between *B. rapa* and *B. nigra*. Plant Breed 123:204–206

Sukno S, Ruso J, Jan CC, Melero-Vara JM, Fernandez-Martinez JM (1999) Interspecific hybridization between sunflower and wild perennial *Helianthus* species via embryo rescue. Euphytica 106:69–78

Taira T, Larter EN (1978) Factors influencing development of wheat-rye hybrid embryos *in vitro*. Crop Sci 18:348–350

Tan ML, Song JK, Deng XX (2007) Production of two mandarin trifoliate orange hybrid populations via embryo rescue with verification by SSR analysis. Euphytica 157:155–160

Tian L, Wang YJ (2008) Seedless grape breeding for disease resistance by using embryo rescue. Vitis 47:15–19

Tian L, Wang YJ, Niu L, Tang DM (2008) Breeding of disease-resistant seedless grapes using Chinese wild *Vitis* spp. I. *In vitro* embryo rescue and plant development. Sci Hort 117:136–141

Tonguç M, Griffiths PD (2004a) Development of black rot resistant interspecific hybrids between *Brassica oleracea* L. cultivars and *Brassica* accession a19182, using embryo rescue. Euphytica 136:313–318

Tonguç M, Griffiths PD (2004b) Transfer of powdery mildew resistance from *Brassica carinata* to *Brassica oleracea* through embryo rescue. Plant Breed 123:587–589

Umehara M, Sueyoshi T, Shimomura K, Nakahara T (2006) Production of interspecific hybrids between *Allium fistulosum* L. and *A. macrostemon* Bunge through ovary culture. Plant Cell Tiss Org Cult 87:297–304

Wen J, Tu JX, Li ZY, Fu TD, Ma CZ, Shen JX (2008) Improving ovary and embryo culture techniques for efficient re-synthesis of *Brassica napus* from reciprocal crosses between yellow-seeded diploids *B. rapa* and *B. oleracea*. Euphytica 162:81–89

White PR (1963) The cultivation of animal and plant cells. Ronald Press, New York, NY, pp 1–228

Williams WM, Verry IM, Ansari HA, Hussain SW, Ullah I, Williamson ML, Ellison NW (2011) Eco-geographically divergent diploids, Caucasian clover (*Trifolium ambiguum*) and western clover (*T. occidentale*), retain most requirements for hybridization. Ann Bot 108:1269–1277

Xu J, Kasha KJ (1992) Transfer of a dominant gene for powdery mildew resistance and DNA from *Hordeum bulbosum* into cultivated barley (*H. vulgare*). Theor Appl Genet 84:771–777

Yanagino T, Sugawara E, Watanabe M, Takahata Y (2003) Production and characterization of an interspecific hybrid between leek and garlic. Theor Appl Genet 107:1–5

Yang HH, Chen XS, Feng BC, Liu HF, Zheng Z (2004) Creating new germplasm by distant hybridization in stone fruits II. Embryo rescue and hybrid identification between plum and apricot. Sci Agri Sinica 37:1203–1207

Yeung EC, Sussex IM (1979) Embryogeny of *Phaseolus coccineus*: the suspensor and the growth of the embryo-proper *in vitro*. Z Pflanzenphysiol 91:423–433

Yoon JB, Yang DC, Do JW, Park HG (2006) Overcoming two post-fertilization genetic barriers in interspecific hybridization between *Capsicum annuum* and *C. baccatum* for introgression of anthracnose resistance. Breed Sci 56:31–38

Yoon JB, Yang DC, Lee WP, Ahn SY, Park HG (2004) Genetic resources resistant to anthracnose in the genus *Capsicum*. J Kor Soc Hort Sci 45:318–323

Yuan HY, Lulsdorf M, Tullu A, Vandenberg A (2011) *In vivo* grafting of wild *Lens* species to *Vicia faba* rootstocks. Plant Genet Res 9:543–548

Zhang GQ, Tang GX, Song WJ, Zhou WJ (2004) Resynthesizing *Brassica napus* from interspecific hybridization between *Brassica rapa* and *B. oleracea* through ovary culture. Euphytica 140:181–187

Zhang GQ, Zhou WJ, Gu HH, Song WJ, Momoh EJJ (2003) Plant regeneration from hybridization of *Brassica juncea* and *B. napus* through embryo culture. J Agron Crop Sci 189:347–350

Zhu PF, Wei YT (2009) Compatibility, production of interspecific F_1 and BC_1 between improved CMS *Brassica campestris* ssp. *pekinensis* and *B. oleracea* var. *acephala*. J Plant Breed Crop Sci 1:265–269

Chapter 5
Horizontal Gene Transfer Through Genetic Transformation

Pooja Bhatnagar-Mathur, Paramita Palit, and K.K. Sharma

Abstract Gene transfer technology in crop plants has tremendous potential to introduce newer and better traits through development of transgenics and broaden the genetic base of crop plants by transferring genes from novel sources overcoming the species and genus barriers. Nevertheless, development of efficient transformation systems remains a prerequisite and might involve many years of exhaustive research. This chapter overviews the different methods of alien gene transfer through genetic transformation and factors affecting efficient transformation across different crop species. A comparative study on *Agrobacterium* and biolistics-mediated transformation including methods for production of marker-free transgenics are described in detail. Addressing the growing concerns over the biosafety issue constraining wider application of GM products in agriculture this chapter also focuses on improved methods of choice with respect to a crop family and also deals with future strategies which can help in further exploiting the existing technologies to develop improved crop varieties which can help to combat poverty, hunger and global agro-climatic changes.

Keywords *Agrobacterium*-mediated transformation • Biolisitcs • Co-transformation • Gene transfer • Marker-free transgenics • Selectable marker • Transgene escape

---

P. Bhatnagar-Mathur, Ph.D. (✉) • P. Palit • K.K. Sharma
Genetic Transformation Laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh 502 324, India
e-mail: p.bhatnagar@cgiar.org

A. Pratap and J. Kumar (eds.), *Alien Gene Transfer in Crop Plants, Volume 1: Innovations, Methods and Risk Assessment*, DOI 10.1007/978-1-4614-8585-8_5,
© Springer Science+Business Media New York 2014

5.1 Introduction

The recent advancement of horizontal gene transfer technology enabled scientists to find a better way to answer problems related to stress response, disease or herbicide resistance or development of tolerance against climate change. It is now feasible to introduce into crop plants genes that have previously been inaccessible to the plant breeder or which did not exist in the crop of interest.

Transgenic technologies have enormous potential to improve crops in a relatively precise way (Barampuram and Zhang 2011). Genes of interest are introduced, often by *Agrobacterium*-mediated transformation, and become integrated at random positions in the genome. Initial experiments involved gene transfer by using *Agrobacterium tumefaciens* (Herrera-Estrella et al. 1983). The development of sophisticated methods later opened the way for an alternative procedure for engineering plants using direct DNA transfer. The protocols for this transfer include particle bombardment (Gan 1989), chemical treatments and electroporation (Bates 1994). However, the unavailibility of efficient transformation methods to introduce foreign DNA (alien gene) can be a substantial barrier to the application of recombinant DNA methods in some crop plants (Bhatnagar et al. 2010).

Despite significant advances over the past decade, the development of efficient transformation methods can take many years of painstaking research (Sharma et al. 2005a, b). The major components for the development of transgenic plants are (1) the development of reliable tissue culture regeneration systems; (2) preparation of gene constructs and transformation with suitable vectors; (3) efficient transformation techniques for the introduction of genes into the crop plants; (4) recovery and multiplication of transgenic plants; (5) molecular and genetic characterisation of transgenic plants for stable and efficient gene expression; (6) transfer of genes to elite cultivars by conventional breeding methods if required; and (7) evaluation of transgenic plants for their effectiveness in alleviating the biotic and abiotic stresses without being an environmental biohazard (Birch 1997). Some of the key characteristics of these components are discussed in this chapter.

5.2 Plant Regeneration in Tissue Cultures

Transformation of plants involves the stable introduction of DNA sequences usually into the nuclear genome of cells capable of giving rise to a whole transformed plant. Transformation without regeneration and regeneration without transformation are of limited value (Bhatnagar et al. 2010). The very basis of regeneration in tissue cultures is the recognition that somatic plant cells are totipotent (i.e., capable of giving rise to a whole plant) and can be stimulated to regenerate into whole plants in vitro, via organogenesis (shoot formation) or somatic embryogenesis, provided they

are given the optimum hormonal and nutritional conditions (Skoog and Miller 1957). Adventitious shoots or embryos are thought to arise from single cells and, thus, provide totipotent cells that can be identified which are both competent and accessible for gene transfer and will give rise directly to nonchimeric transformed plants. Transformation techniques reliant on plant regeneration from in vitro-cultured tissues have been described for many crop species (Lindsey and Jones 1989; Birch 1997).

5.3 Transformation Vectors

Most vectors used for the genetic transformation of plants carry 'marker' genes that allow the recognition of transformed cells, by either selection or screening. These genes are dominant, usually of microbial origin, and placed under the control of strong and constitutive, eukaryotic promoters, often of viral origin (Birch 1997). The most popular selectable marker genes used in plant transformation vectors include constructs providing resistance to antibiotics such as kanamycin, chloramphenicol and hygromycin and genes that allow growth in the presence of herbicides such as phosphinothricin, glyphosate, bialaphos and several other chemicals (Wilmink and Dons 1993).

For successful selection, the target plant cells must be susceptible to relatively low concentrations of the antibiotic or the herbicide in a non-leaky manner. Screenable marker 'reporter genes' have also been developed from bacterial genes coding for easily assayed enzymes, such as chloramphenicol acetyl transferase (CAT), b-galactosidase, b-glucuronidase (GUS), luciferase (LUX), green fluorescent protein (GFP), nopaline synthase and octopine synthase (Herrera-Estrella et al. 1983, Reichel et al. 1996). The utility of any particular marker gene construct as a transformation marker varies depending on the plant species and explant involved. To date kanamycin resistance (Reiss et al. 1984) is the most widely used selectable marker phenotype, and b-glucuronidase (Jefferson et al. 1987) is the most widely used screenable marker.

Most commonly used plant transformation vectors have features required for various recombinant DNA manipulations that include multiple unique restriction sites, bacterial origins of replication and prokaryotic selectable markers for plasmid selection and maintenance in *Escherichia coli* (e.g. antibiotic resistance). In addition, these vectors contain specific selectable marker genes engineered for expression in plants that may be used directly as transformation vectors in physical DNA delivery strategies such as particle bombardment. However, for *Agrobacterium*-mediated gene transfer, these vectors need additional features such as wide host range replication and transfer functions to allow conjugation from *E. coli* to *Agrobacterium* and plasmid maintenance in both bacterial hosts (Klee et al. 1987).

5.4 Methods of Plant Gene Transfer

5.4.1 *Agrobacterium*-Mediated Gene Transfer

Agrobacterium tumefaciens is a soil bacterium that leads to gall formation at the wound sites of many dicotyledonous plants. The tumour inducing capability is due to the presence of a large Ti (tumour inducing) plasmid in virulent strains of *Agrobacterium.* Likewise, Ri (root-inducing) megaplasmids are found in virulent strains of *A. rhizogenes*, the causative agent of 'hairy root' disease. The molecular biology of Ti and Ri plasmids and of crown gall and hairy root induction have been studied in great detail (Klee et al. 1987; Zambryski 1992). The number of plant species transformed by *Agrobacterium* vectors has increased steadily over the past few years, and representatives of many taxonomically diverse genera have proved amenable to transformation (Dale et al. 1993). This success can mainly be ascribed to the improvements in tissue culture technology, particularly adventitious shoot regeneration in the crop plants concerned. *Agrobacterium*-mediated transformation in plants has been carried out across a vast range of plant species by using both tissue culture-dependent transformation as well as tissue culture-independent transformation (non-tissue culture-based) techniques (Keshamma et al. 2008; Rao et al. 2012).

The important requirements for *Agrobacterium*-mediated transformation firstly include the production of some active compounds like acetosyringone by the explants in order to induce the *vir* genes present on the Ti plasmid and then the induced *Agrobacteria* must have access to competent plant cells that are capable of regenerating adventitious shoots or somatic embryos at a reasonable frequency (Barghchi 1995). There is evidence to suggest that for gene transfer to occur, cells must be replicating DNA or undergoing mitosis (Moloney et al. 1989; Sharma et al. 1990). The majority of transformation experiments utilise either freshly explanted tissue sections or protoplasts in the process of reforming a cell wall and entering cell division or callus and suspension-cultured cell clumps wounded by chopping or pipetting and stimulated into rapid cell division by the use of nurse cultures (Draper et al. 1988). The adventitious shoot production in vitro is most commonly employed in most systems of genetic transformation.

5.4.1.1 Role of *Agrobacterium*-Related Factors in Alien Gene Transfer

Plant-specific factors, such as compounds (phenolics) that induce the expression of *Agrobacterium vir* genes, are necessary for efficient transformation (Stachel and Zambryski 1989). Virulence-inducing phenolic compounds were first described by Bolton et al. (1986) and are limited to dicolyledonous plants (Smith and Hood 1995). Although these have been comprehensively reviewed (Gheysen et al. 1998; Gelvin 2000), transfer and integration process of T-DNA is still not fully understood.

Till date, several key factors involved in *Agrobacterium*-mediated transfer have been described (Pradhan et al. 2012; Guo et al. 2012). Impact of these factors on

transformation efficiency was modified by using a large number of *Agrobacterium* strains (Klee 2000), binary vectors (Hoekema et al. 1983), disarmed plasmids such as disarmed version of p*TiBo542* (Hood et al. 1986) and use of super-binary vector with a fragment containing the *virB*, *virC* and *virG* genes (from p*TiBo542*). Multiple T-DNAs were delivered to plant cells either from a mixture of strains or from a single strain and segregation of one T-DNA from others observed in various occasions (McKnight et al. 1987, De Block and Debrouwer, 1991). In another approach Komari et al. (1996) co-transformed tobacco and rice with unique plasmids carrying two separate T-DNAs and were able to separate them in successive generations by Mendelian segregation.

Addition of phenolic compounds, particularly acetosyringone, enhances the induction of the *Agrobacterium vir* genes, during bacteria/plant co-cultivation (Vijayachandra et al. 1995). Hence it was recognised as a key for successful transformation in rice (Hiei et al. 1994). Other inducing factors are low pH (Godwin et al. 1991), temperature (Dillen et al. 1997) and high osmotic pressure (Usami et al. 1988). It has been observed that certain carbohydrates in the presence of 100 μM acetosyringone did not have any significant synergistic effect (Hiei et al. 1997). Wounding of targeted tissue prior to co-cultivation enhanced *Agrobacterium* transformation frequencies by microprojectile bombardment (Bidney et al. 1992). However, inoculation of *Agrobacterium* after plasmolysis alone gave an even better transient expression compared to the combination of plasmolysis and bombardment (Uzé et al. 1997). Genotype and type of tissue to be transformed, composition of culture media and elimination of *Agrobacterium* after co-cultivation further influence the efficient production of stable transformants in plants (Nauerby et al. 1997).

5.4.1.2 Factors Affecting *Agrobacterium*-Mediated Transformations

The transfer of T-DNA and its integration into the plant genome are influenced by several *A. tumefaciens* and plant tissue-specific factors. These include plant genotype, explant, vectors-plasmid, bacteria strain, addition of *vir*-gene-inducing synthetic phenolic compounds, culture media composition, tissue damage, suppression and elimination of *A. tumefaciens* infection after co-cultivation (Nauerby et al. 1997; Klee 2000).

Osmotic Treatment

Osmotic treatment for enhancement of *Agrobacterium*-mediated transformation largely depends upon the species. However, plasmolysis with sucrose (292 mM) improved T-DNA delivery into precultured immature embryos of rice (Uzé et al. 1997) and was later used extensively later on in development of transgenic plants (Lucca et al. 2001). Extensive use of sucrose and glucose transformation did not describe any effect of osmotic medium on T-DNA delivery and stable transformation in rice and maize (Hiei et al. 1994; Zhao et al. 2001; Frame et al. 2002) and in wheat (Cheng et al. 2003).

Preconditioning and Co-cultivation Time

Co-cultivation of explants with *A. tumefaciens* has made possible the use of some explants, which were hitherto recalcitrant for transformation. Optimising the preconditioning time (72 h) and co-cultivation time with *A. tumefaciens* (48 h) increased the transformation efficiency in canola (Cadoza and Stewart 2003) and in Chinese cabbage (Zhang et al. 2000).

Desiccation of Explants

A significant factor that enhances transformation of crop species is desiccation of explants prior to or post *A. tumefaciens* infection. Arencibia et al. (1998) reported that air-drying sugarcane suspension cells prior to inoculation under laminar flow for 15–60 min slightly improved T-DNA delivery and subsequently increased transformation efficiency. Similarly in rice, air-drying calli derived from suspension cultures for 10–15 min increased the transformation efficiency by tenfold or more (Urushibara et al. 2001). Desiccation of precultured immature embryos, suspension culture cells and embryonic calluses of wheat and embrogenic calluses of maize greatly enhanced T-DNA delivery and plant tissue recovery after co-culture, leading to increased stable transformation frequency (Cheng et al. 2003). This treatment has also improved T-DNA delivery in dicot species such as soybean suspension cells based on preliminary study (Cheng and Fry 2000). Although the molecular mechanism of desiccation during co-culture remains unclear, it is known that desiccation suppresses the growth of *Agrobacterium* similar to the effect observed with silver nitrate (Opabode 2006).

Anti-necrotic Treatments

Anti-necrotic mixtures for pre-induction have shown to be important for reducing oxidative burst. Treatment of meristem explants of sugarcane and rice with medium containing 15 mg/1 ascorbic acid, 40 mg/1 cysteine and 2 mg/1 silver nitrate improved the transformation efficiency and explant viability (Enriquez-Obregon et al. 1999). Inclusion of silver nitrate in co-culture medium enhanced stable transformation in maize (Armstrong and Rout 2001; Zhao et al. 2001). It significantly suppresses the *Agrobacterium* growth during co-culture without compromising T-DNA delivery and subsequent T-DNA integration, facilitating plant cell recovery and increased efficiency of transformation (Cheng et al. 2003). Inclusion of cysteine in the co-culture medium led to an improvement in both transient GUS expression in target cells and a significant increase in stable transformation frequency in maize (Somers et al. 2003).

Temperature

The effect of temperature during co-culture on T-DNA delivery was first reported in dicot species (Dillen et al. 1997). The optimal temperature for stable transformation

should be evaluated with each specific explant and *Agrobacterium* strain involved (Salas et al. 2001; Alimohammadi and Bagherieh-Najjar 2009). A temperature of 22 °C was found to be optimal for T-DNA delivery in tobacco leaves (Dillen et al. 1997). However, in another study the highest number of transformed plants were obtained in tobacco at 25 °C, even though 19 °C was optimal for T-DNA delivery (Salas et al. 2001). In monocots, the co-culture temperature for most of the crops ranged from 24 to 25 °C, and in some cases, 28 °C was used for co-culture (Rashid et al. 1996; Enriquez-Obregon et al. 1999; Hashizume et al. 1999). The effect of lower temperature (23 °C) on T-DNA delivery and stable transformation was also evaluated, and highest transient GUS expression (64 %) was observed at 22 °C in garlic (Kondo et al. 2000). In maize, higher transformation frequency was observed at 20 °C than at 23 °C (Frame et al. 2002). In another study, transgenic maize plants were obtained by co-culture of the immature embryos at 20 °C followed by 28 °C subculture (Gordon-Kamm et al. 1990). The optimal temperature for both T-DNA delivery and stable transformation was 23–25 °C for wheat and 23 °C for maize (Frame et al. 2002).

Surfactants

Including surfactants such as Silwet L77 and Pluronic acid F68 in inoculation medium greatly enhanced T-DNA delivery in immature embryos of wheat (Cheng et al. 1997). These surfactants may enhance T-DNA delivery by aiding *A. tumefaciens* attachment and/or by elimination of certain substances that inhibit this attachment. Their addition in the inoculation medium plays a role similar to vacuum infiltration, i.e. facilitating the delivery of *A. tumefaciens* cells to closed ovules (primary target for transformation of *A. thaliana* (Ye et al. 1999; Desfeux et al. 2000)). The surfactant Silwet L77 was shown to be useful to the success of the floral dip method of *Arabidopsis thaliana* transformation (Dehestani et al. 2010).

Inoculation and Co-culture Medium

Culture medium components like sugar, plant growth regulators and *vir* induction chemicals are important factors that affect the transformation frequency. MS (Murashige and Skoog 1962) or a modified MS-based medium has shown to be suitable for inoculation and co-culture (Dong et al. 1996; Enriquez-Obregon et al. 1999; Lucca et al. 2001). The modified N6 medium (Chu et al. 1995) containing 2,4-dichlorophenoxyacetic acid (2,4-D) was shown to be suitable for co-culture in rice. Transformation of maize immature embryo using LS-based (Linsmaier and Skoog 1965) medium and N6-based medium failed to generate transformed plants (Ishida et al. 1996). However, addition of silver nitrate in N6-based medium for inoculation and co-culture of immature embryos resulted in regeneration of transgenic plants in maize (Zhao et al. 2001). Similarly, addition of $CaCl_2$ in the medium has increased transformation efficiency in barley (Kumlehn et al. 2006). One-tenth MS salt

strength enhanced transient GUS expression tenfold over full-strength salts in barley (Ke et al. 2002). Furthermore, the distribution of cells expressing the GUS gene within each set of immature embryos was clearly altered, showing significantly more cells on the scutellar surface expressing GUS. Reduction in the salt strength of the inoculation and co-culture media was shown to be useful in development of transgenics of canola (Fry et al. 1987), wheat (Cheng et al. 1997) and maize (Armstrong and Rout 2001; Khanna and Daggard 2003). Use of *vir* induction chemicals improved the transformation efficiency in most of the crops (Cheng et al. 1997; Zhao et al. 2000; Kumlehn et al. 2006). However in some other cases, explants of monocot species could be efficiently transformed without the aid of external *vir* induction chemicals for special treatment (Enriquez-Obregon et al. 1999; Cheng et al. 2003).

Antibiotics

Antibiotics such as cefotaxime, carbenecillin and timentin have been used regularly in *Agrobacterium*-mediated transformation (Cheng et al. 1997; Bottinger et al. 2001; Sunikumar and Rathore 2001). Though initially cefotaxime worked well in rice and maize, later on it was observed that its use had a detrimental effect to maize Hi II callus (Ishida et al. 1996). Hence the use of carbenicillin has become the antibiotic of choice in reports of *Agrobacterium*-mediated transformation of wheat and maize in subsequent studies (Cheng et al. 2003, Zhang et al. 2003). On the other hand, 100 mg/l kanamycin was economical and improved the transformation efficiency in white spruce by enrichment of transformed tissue in budforming callus (Le et al. 2001) and increased the proportion of positively transformed shoots during subculture on kanamycin-containing medium in peanut and pigeonpea (Sharma and Anjaiah 2000; Thu et al. 2003).

Selectable Marker

Genes encoding for hygromycin phosphotransferase (*hpt*), phosphinothricin acetyltransferase (*pat* or *bar*) and neomycin phosphotransferase (*npt*II) are most widely used selectable markers for transformation. These marker genes work well under the control of constitutive promoters such as the *35S* promoter from cauliflower mosaic virus and the ubiquitin promoter from maize for selection of transformed cells. In *Asparagus* and banana, the *npt*II gene under the control of the nopaline synthase promoter has been used successfully to select stable transformants with kanamycin (May et al. 1995; Limanton-Grevet and Jullien 2001). The positive selectable marker phosphomannose isomerase (PMI) was first used for *Agrobacterium*-mediated transformation of sugar beet and was recently used to enhance transformation of sorghum (Joersbo et al. 1998; Gao et al. 2005). Introns were inserted into the coding region of *hpt* for enhancing transgene expression in monocot species (Simpson and Filipowcz 1996). Besides improving transformation frequency in rice, this modification in the selectable marker reduced copy numbers

of the marker gene, enabled better control of *Agrobacterium* growth during the transformation (Wang et al. 1997) and enhanced stable transformation (Wang et al. 2001). Glyphosate-insensitive plant 3-enolpyruvylshikimate-5-phosphate synthase (EPSPS) genes, the bacterial CP4 gene or a bacterial gene that degrades glyphosate, i.e. glyphosate oxidoreductase (GOX) gene, have also been used as selectable marker genes to generate transgenic plants in wheat and maize (Armstrong and Rout 2001; Howe et al. 2002; Hu et al. 2003).

5.4.2 *Modified Methods* of *Agrobacterium-Mediated Gene Transfer*

5.4.2.1 Sonication-Assisted *Agrobacterium*-Mediated Transformation

An important modification in *Agrobacterium*-mediated transformation involves subjecting the plant tissue to brief periods of ultrasound in the presence of *Agrobacterium*. Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) treatment produces a large number of small and uniform wounds throughout the tissue, allowing easy access to the *Agrobacterium*, resulting in improved transformation efficiency in several different plant tissues including immature cotyledons, leaf tissue, suspension cultures and somatic and zygotic embryos. It was reported to increase transformation rates in those species which are more recalcitrant to *Agrobacterium*-mediated transformation (Trick and Finer 1997).

Tissue culture-independent transformation systems have also been demonstrated in various crops such as soybean (Chee et al. 1989), Arabidopsis (Feldmann and Marks 1987), sunflower (Rao and Rohini 1999), safflower (Rohini and Rao 2000a) and peanut (Rohini and Rao 2000b). Arabidopsis seeds infected with *Agrobacterium* and allowed to grow into mature plants in vivo resulted in about 1 % transformation frequency. Inoculation of *Agrobacterium* onto wounded sites arising from cutting away inflorescences of *Arabidopsis* yielded transformed seeds from newly emerging inflorescences (Chang et al. 1994; Katavic et al. 1994). This has also been used to generate transgenics in groundnut (Rohini and Rao 2000b).

5.4.2.2 Floral Dip Method

In this method, *Agrobacterium* is directly applied to floral tissues and thus eliminates possibility of generation of somaclonal variations due to the bypass of tissue culture techniques (Clough et al. 1998). In *Arabidopsis*, studies demonstrated the use of female gametophytes of immature flowers as targets of floral-dip transformation (Ye et al. 1999; Desfeux et al. 2000). This method requires considerably less time and effort than vacuum infiltration and is greater in yield. *Agrobacterium*-based floral dip transformation method, requiring no vacuum infiltration step, reported transformation efficiencies up to 0.8 % (Liu et al. 2012).

5.4.2.3 Vacuum Infiltration Method

The vacuum infiltration method of transformation has been applied mostly in monocot crops in order to avoid both in vitro culture and regeneration steps during transformation. The cells of a plant when subjected to a vacuum environment establish more intimate contact with *Agrobacterium*. This method was used to obtain stable transgenics in *Medicago truncatula* (a model legume plant) (Trieu et al. 2000).

5.4.2.4 Agrolistics

The agrolistics approach combines the advantages of efficient biolistic delivery and the precision of the *Agrobacterium* T-DNA insertion mechanism, minimising the regions of homology contributing to genetic and/or epigenetic instability (Hansen and Chilton 1996). By combining features of *Agrobacterium*-mediated transformation it is possible to achieve relatively predictable inserts in plants that are not normally transformable using *Agrobacterium*. Agrolistic transformation allows integration of the gene of interest without the undesired vector sequence, using plant expression cassettes for *virD1* and *virD2* genes co-delivered with a vector containing T-DNA border sequences flanking a gene of interest, resulting in production of transformants without the extraneous vector DNA as a result of T-DNA border cleavage by *virD1* and *virD2* gene products (Sharma et al. 2005a, b).

5.4.3 Biolistics-Mediated Gene Transfer

The invention of the particle bombardment technique (Sanford et al. 1987) was a major breakthrough in plant transformation as it has enabled the genetic engineering of species not amenable to *Agrobacterium* or protoplast-based transfer techniques. Based on acceleration, microscopic tungsten (Russel et al. 1992) or gold particles coated with DNA can be propulsed into practically all kinds of tissues (Tomes et al. 1990; Ritala et al. 1994; Zhong et al. 1996). It has been used to develop the transgenic cereal plants in wheat (Vasil et al. 1992), oat (Somers et al. 1992), barley (Wan and Lemaux 1994) and rye (Castillo et al. 1994).

5.4.3.1 Factors Affecting Biolistics-Mediated Gene Transfer

Several factors have been found to influence the applicability and efficiency of biolistic gene transfer. The factors related with tissue culture techniques include genotype (Koprek et al. 1996), type and age of bombarded explants (Armaleo et al. 1990), culture period prior to and after gene transfer (Rasco-Gaunt et al. 1999), culture medium composition (Barro et al. 1998) and osmotic pretreatment (Vain et al. 1993). Concerning the biolistic device, the applied acceleration pressure

(Rasco-Gaunt et al. 1999); the adjustable distances between rupture disc, macrocarrier, stopping screen and target plate (Koprek et al. 1996; Rasco-Gaunt et al. 1999); the vacuum pressure in the bombardment chamber (Rasco-Gaunt et al. 1999); number of bombardments (Lonsdale et al. 1990) as well as size and density of micro-particles (Altpeter et al. 1996; Rasco-Gaunt et al. 1999); DNA/micro-particle mixing protocols (Perl et al. 1992) and physical configuration of transforming DNA (Nandadeva et al. 1999; Fu et al. 2000) are factors to be optimised. Several attempts to establish or improve transformation protocols focused on transient GUS expression without consideration of the regeneration response of the bombarded tissues (Chibbar et al. 1991; Bilang et al. 1993). However, particle bombardment, especially of recalcitrant species, can have severe effects on the regeneration capability of cultures. Optimised protocols for generating transgenic plants should therefore not be based exclusively on transient gene expression assays (Nandadeva et al. 1999); adjustment of bombardment parameters to maintain the shoot regeneration ability and allow the recovery of stable transformants is recommended (Altpeter et al. 1996).

5.4.4 Other Methods of Gene Transfer

Other DNA delivery protocols like macroinjection (Soyfer 1980; Zhou et al. 1983), the use of silicon carbide whiskers (Wang et al. 1995; Petolino et al. 2000) and ultrasound- (Joersbo and Brunstedt 1990) or laser-mediated gene transfer (Weber et al. 1988) are of rather theoretical importance and have been extensively reviewed by Rasco-Gaunt et al. (1999).

5.5 Marker-Free Plants: The Most Relevant Answers to Biosafety-Related Issues

Selectable marker gene (SMG) and reporter genes play the most crucial role in transferring foreign genes and are almost always present in engineered DNA plasmids used for genetic transformation of plant tissue (Lee and Gelvin 2008). It is only the presence of SMG that serves as a weapon for transformed cells to tolerate a lethal exposure to antibiotic and herbicide, and the desired gene can safely grow and regenerate into the plants (Lee and Gelvin 2008). Selectable markers and visible marker reporter genes rarely affect the studied trait of interest, but provide a powerful tool in determining the success of the transformation events or identification of transformation events before the gene of interest can be identified in the culture (Sheen et al. 1995). However there is a need to free transgenic plants from these markers due to harmful effects to human. Therefore the following strategies have been used widely to remove such markers.

5.5.1 Co-transformation

The co-transformation method is used to eliminate the marker gene from the nuclear genome and involves transformation that targets insertion at two different plant genome loci. Co-transformation studies utilise the strategy to load the selectable and target genes between the same pair of borders or loaded into separate T-DNAs, which are expected to segregate independently in a Mendelian fashion (Ramana Rao et al. 2011). The three methods used in the co-transformation system include (1) two different vectors carried by different *Agrobacterium* strains (De Neve et al. 1997) and biolistic introduction of two plasmids in the same tissue (Shiva Prakash et al. 2009; Kumar et al. 2010); (2) two different vectors in the same *Agrobacterium* cell (Sripriya et al. 2008) and (3) two T-DNAs borne by a single binary vector (2 T-DNA system) (Miller et al. 2002). This has the following advantages.

1. The conventional unmodified *Agrobacterium*-mediated gene transfer methods have high adaptability and easier handling of the binary vectors (Tuteja et al. 2012).
2. This method depends on the co-transformation efficiency which ranges from 30 to 50 % and the independent integration of T-DNA into the plant genome, which is acceptable for practical applications (McCormac et al. 2001). Recently, in rice, high transformation frequency (86 %) was achieved through genetic separation in four out of ten primary co-transformants that were forwarded to the T1 generation (Sripriya et al. 2011).

5.5.2 Multi-Autotransformation Vector System

The multi-autotransformation (MAT) vector system represents a highly sophisticated approach for the removal of nuclear marker genes (Ebinuma et al. 1997). It is a unique transformation system that is based on morphological changes caused by oncogene [the isopentenyl transferase (*ipt*) gene] or rhizogene (the *rol* gene) of *A. tumefaciens* (Tuteja et al. 2012). The *ipt*-type MAT system has been considered better for successful generation of marker-free transgenic plants (Saelim et al. 2009). This system utilises the *ipt* gene as morphological marker for visual selection of transgenic lines. The extreme shooty phenotype (ESP) of transgenic lines is lost due to the removal of *ipt* gene mediated by the yeast recombinase/recognition sites R/RS system. As a result, phenotypically normal shoots, considered marker-free transgenic plants, could be obtained. The *ipt* marker gene has been efficiently used as selectable marker gene for obtaining marker-free plant in several crops (Khan et al. 2010a, b). Rol-type MAT vector (pMAT101) containing *lacZ* gene as a model gene and the removable cassette with *GUS* gene in the T-DNA region were used to produce morphologically normal transgenic *Kalanchoe blossfeldiana* pollen, employing *rol* gene as the selectable marker gene and *gus* gene as a reporter

gene (Thirukkumarana et al. 2010). This vector has been tried in *Antirrhinum majus* (Cui et al. 2001), tobacco (Ebinuma and Komamine 2001), white poplar (Zelasco et al. 2007) and *Petunia hybrida* (Khan et al. 2010c). Genetic transformation of an elite white poplar genotype (*Populus alba* L., cv. 'Villafranca') was performed with MAT vectors carrying the *ipt* and *rol* genes from *A. tumefaciens* spp. as morphological markers. The occurrence of abnormal *ipt* and *rol* phenotypes allowed the visual selection of transformants (Zelasco et al. 2007). *A. tumefaciens* strain EHA105 harbouring a Rol-type MAT vector, pMAT101, was used to produce morphologically normal transgenic *Petunia hybrida* 'Dainty Lady' employing *rol* gene as the selection marker gene. The *lacZ* gene was used as a model GOI (Khan et al. 2010c).

5.5.3 Site-Specific Recombination

The site-specific recombination methods in plants have been developed to delete selection markers to produce marker-free transgenic plants or to integrate the transgene into a predetermined genomic location to produce site-specific transgenic plants (Nanto and Ebinuma 2008). The three well-known site-specific recombination systems discussed below are used for the elimination of selection marker gene.

5.5.4 Cre/Lox Site-Specific Recombination System

The Cre/loxP (CLX) system consists of two components: (a) two loxP sites each consisting of 34 bp inverted repeats cloned in direct orientation flanking a DNA sequence and (b) the *cre* gene encoding a 38 kDa recombinase protein that specifically binds to the loxP sites and excises the intervening sequence along with one of the loxP sites. A number of studies describes the successful utilisation of CLX system including *Arabidopsis thaliana* (Zuo et al. 2001), *Nicotiana benthamiana* (Gleave et al. 1999), *Zea mays* (Zhang et al. 2003) and *Oryza sativa* (Hoa et al. 2002; Sreekala et al. 2005).

The specificity of the enzyme for its 34 bp recognition sequence is one of the major advantages of Cre/lox system because insertion and excision of genes with precision in the plant genome without a site-specific recombination system are difficult (Tuteja et al. 2012). However, use of this system for marker gene removal requires re-transformation and outcrossing approaches that are laborious and time consuming (Dale and David 1991). Several approaches were developed to overcome these shortcomings, including the use of some chemical inducers (Zhang et al. 2006) and heat shock (Cuellar et al. 2006). Marker-free transgenic tomato plants expressing *Cry1Ac* were obtained by using a chemically regulated Cre/lox-mediated site-specific recombination system (Zuo et al. 2001; Zhang et al. 2006).

Lin et al. (2008) reported a chemical induction method for creating selectively terminable transgenic rice using benzothiadiazole (bentazon), a herbicide used for weed control in major crops like rice, maize, wheat, cotton and soybean. Similarly, Ma et al. (2009) used salicyclic acid-inducible Cre–loxP recombination system to develop marker-free transgenic tomato.

5.5.5 Flippase/Flippase Recombination Target Recombination System

Nandy and Srivastava (2011) reported the use of flippase (FLP)/flippase recognition target (flp) system for efficient targeting of foreign gene into the engineered genomic site in rice. In the FLP/frt site-specific system, the FLP enzyme efficiently catalyses recombination between two directly repeated FLP recombination target (frt) sites, eliminating the sequence between them (Tuteja et al. 2012). By controlled expression of the FLP recombinase and specific allocation of the frt sites within transgenic constructs, the system can be applied to eliminate the marker genes after selection (Cho 2009). This system has been used to generate marker-free salt-tolerant transgenic maize plants constitutively expressing AtNHX1, a Na(+)/H(+) antiporter gene from *A. thaliana* (Li et al. 2010). An oxidative stress-inducible FLP gene was used successfully to excise antibiotic-resistance genes from transgenic plants (Woo et al. 2009). Two site-specific recombination systems, Cre/lox and FLP/frt, were tested for marker gene removal and targeted gene transfer in a *Populus* (Fladung et al. 2010) and observed to be useful for removal of marker genes. Combining both site-specific recombination systems, a strategy is suggested for targeted transgene transfer and removal of antibiotic marker genes.

5.5.6 R/RS Recombination System

The MAT vectors consist of yeast site-specific recombination R/RS system to excise the DNA fragment and the *ipt* gene positioned between two directly oriented recombination sites (Araki et al. 1987). The R/RS system comprises an R recombinase gene and two RS recombination site sequences. The *ipt* combined with the *'R'* gene is placed within two directly oriented recognition sites to remove it from transgenic cells after transformation. The improved MAT vector is used to generate marker-free transgenic plants efficiently. A new binary vector for *A. tumefaciens*-mediated transformation, pHUGE-Red, was developed (Untergasser et al. 2012). This vector enables modular cloning of large DNA fragments by employing Gateway technology and contains DsRED1 as visual selection marker. However, an R/Rs-inducible recombination system was included allowing subsequent removal

of the selection markers in the newly generated transgenic plants. This strategy allowed successful transfer of eight genes essential for *Medicago truncatula* (Untergasser et al. 2012).

5.5.7 Transposon-Based Marker Methods

Transposon-mediated repositioning of a transgene of interest has been proposed as an alternative for generating a wide range of expression levels in selectable marker gene-free transgenic plants (Yoder and Goldsbrough 1994). Two transposon-mediated strategies have been developed to generate marker-free transgenic plants. The first strategy involves intragenomic relocation of transgene of interest after *Agrobacterium*-mediated transformation and its subsequent segregation from the selectable marker in the progeny (Goldsbrough et al. 1993). The second involves excision of the marker gene from the genome (Ebinuma et al. 1997). Though maize Ac/Ds transposable element has been used in the above strategies, similar approach can be adopted to other autonomous transposable elements. In 2012, Li and Charng developed an improved strategy involving insertion of the end of the inducible transposon in an intron of a target gene for subsequent removal of its function in transgenic plants. Salicylic acid-induced transposition of COKC transposon, which led to both marker gene and transposase gene breakages in exons, was analysed in single-copy transgenic rice plants. It has been observed that the COKC element exhibits the potential as a tool to create 'marker-off' (marker free) transgenic plants (Li and Charng 2012). However, its application is limited for selectable marker gene without native introns, e.g. hygromycin- or kanamycin-resistant genes. Therefore in order to expand the application of the 'marker-off' transgenic system, an artificial intron containing one end of the transposon has been generated (Li and Charng 2012), and as the result successful transgenic plants were obtained after screening with the selection agent. Thus it indicated the use of an inducible transposon for 'cleaner' plant biotechnology (Li and Charng 2012).

5.5.8 Chemical-Inducible System

The chemical-inducible CLX vector system benefits from a particularly regulated system of chemical induction (Sreekala et al. 2005). It is used in vegetatively propagated and other crop species (Tuteja et al. 2012). The strategy utilises the CLX recombination system keeping it under the control of estrogen receptor-based transactivator XVE. Upon induction by β-estradiaol, the selection marker gene fused with Cre recombinase, flanked by two lox sites and autoexcised from the genome (Zuo et al. 2001). Marker-free transgenic tomato expressing *cry1AC* was obtained by using the above system. A chemical-induced method for creating

selectively terminable transgenic rice using benzothiadiazole herbicide (bentazon) has already been discussed in the section of Cre/lox site-specific recombination system (Lin et al. 2008).

5.5.9 Heat-Inducible System

Transgenic tobacco has been developed using FLP/frt recombinase system in which the expression of FLP was tightly under the control of heat-shock protein (HSP) (Shan et al. 2006). Heat-inducible strategy for the elimination of selection marker gene was also used in vegetatively propogated plants like potato (Cuellar et al. 2006) and seed-producing plants like tobacco (Wang et al. 2005). The disadvantage of this method is that when autoexcision constructs are used, the recombinase can be activated by a chemical compound or by a heat shock in the shoots and seeds or during a subculture step and an extra regeneration step. The latter possibility lengthens the time to obtain marker-free transgenic plants and can introduce (additional) somaclonal variation (Tuteja et al. 2012).

5.5.10 Positive Selection System

A better way to select and identify the genetically modified cell is through the positive selection system. The GUS gene is widely used as a reporter gene in transgenic plants. A glucuronide derivative of benzyladenine (benzyladenine N-3-glucuronide) is used as a selective agent and being in inactive form does not have any effect on the non-transformed cells. However, there are only a few reports concerning the successful use of this system in the effective recovery of transgenic plants (Joersbo and Okkels 1996; Okkels et al. 1997).

The *manA* gene codes for the PMI enzyme (EC 5.3.1.8) and is isolated from *Escherichia coli*. Because mannose is used as the sole carbohydrate source for the transformed cells this selection system is immediate and extremely efficient (Joersbo et al. 1998). Those species which are extremely sensitive to mannose such as sugar beet, maize, wheat, oat, barley, tomato, potato, sunflower, oilseed rape and pea have been successfully transformed by the use of mannose as a selective agent (Joersbo et al. 1998; Wang et al. 2000). Use of positive selection system was found at least ten times more efficient than the traditional protocols based on the use of kanamycin as selection agent (Wright et al. 2001). Similarly a positive selection system has also been developed using the xylose isomerase gene (xylA) isolated from *Thermoanaerobacterium thermosulfurogenes* or from *Streptomyces rubiginosus*, as selection marker gene (Haldrup et al. 1998), and used in development of transgenic plants of potato, tobacco and tomato. The DOG R1 gene encoding 2-deoxyglucose-6-phosphate phosphatase (2-DOG-6-P) was used to develop a positive selection system for tobacco and potato plants (Kunze et al. 2001).

5.5.11 Negative Selection System

Negative selectable markers are of two types: (a) conditional negative selectable marker and (b) non-conditional negative selectable marker (e.g. diphtheria toxin). MYMV TrAP is a good non-conditional negative selectable marker for developing marker-free transgenic plants (Ramana Rao and Veluthambi 2010). It is also possible to apply negative selection after a positive selection using one marker gene. Use of *tms2* gene is the first conditional selective marker gene, which is used in tobacco (Depicker et al. 1988) and *Arabidopsis* (Karlin-Neumann et al. 1991). Other conditional markers proven to be effective in dicots are *aux2* in cabbage (Beclin et al. 1993), the *HSV-tk* gene in tobacco (Czako and Marton 1994), a bacterial cytochrome P450 mono-oxygenase gene in tobacco (O'Keefe et al. 1994) and *Arabidopsis* (Tissier et al. 1999) and *codA* in *Arabidopsis* (Kobayashi et al. 1995) and tobacco (Schlaman and Hooykaas 1997).

5.5.12 Autoexcision Strategy

Autoexcision strategy is used to eliminate selection marker gene from the plant genome, controlled by pollen- and/or seed-specific promoters. Highly efficient autoexcision of selective markers has been reported to be successful in tobacco (Mlynarova et al. 2006; Luo et al. 2007) and in rice (Bai et al. 2008). The novel marker-free approach mediated by the *Cre–lox* recombination system and the *Cre* gene was under the control of floral specific promoter *OsMADS45*. The marker-free transgenic plants of *A. thaliana* were developed by using a germline-specific autoexcision vector containing a *Cre* recombinase gene under the control of a germline-specific promoter (*APETALA1* and *SOLO DANCERS* genes from *Arabidopsis*). However, this strategy is not useful in the vegetatively propagated plants (Verweire et al. 2007).

5.6 Gene Transfer for Stress Tolerance in Crop Species

Development of genetically engineered plants by the introduction and/or overexpression of selected genes seems to be a viable option to hasten the breeding of 'improved' plants against various biotic and abiotic stresses. It is a faster way to achieve transgenesis when genes of interest are originated from cross barrier species, distant relatives or non-plant sources. Several traits associated with resistance to various stresses have been introgressed and tested in transgenic plants for improving stress tolerance in plants (Bhatnagar et al. 2010).

5.6.1 Tolerance to Abiotic Stresses

Drought is one of the most significant environmental stresses on world agricultural production, and enormous efforts are being made by plant scientists to improve crop yields in the face of decreasing water availability. The genes that encode enzymes for the synthesis of selected osmolytes have been used to develop osmoprotection in plants (Bray 1993). This has resulted in a profusion of reports involving osmoprotectants such as glycine-betaine (Ishitani et al. 1997; Sakamoto et al. 2000; McNeil et al. 2000) and proline (Nanjo et al. 1999; Yamada et al. 2005). Also, a number of 'sugar alcohols' (mannitol, trehalose, myo-inositol and sorbitol) have been targeted for the engineering of compatible solute overproduction, thereby protecting the membrane and protein complexes during stress (Pilon-Smits et al. 1995; Garg et al. 2002; Cortina and Culianez-Macia 2005; Gao et al. 2000). Similarly, transgenics engineered for the overexpression of polyamines have also been developed (Waie and Rajam 2003; Capell et al. 2004).

LEA proteins are high-molecular-weight proteins found in abundance during late embryogenesis and accumulate during seed desiccation in response to water stress (Galau et al. 1987). Transgenic melon (Borda's et al. 1997) and tomato (Gisbert et al. 2000) plants expressing the *HAL1* gene showed a certain level of salt tolerance due to retention of more K+ under salinity stress. Overexpression of *AtCLCd* gene, which is involved in cation detoxification, and *AtNHXI* gene which is homologous to *NhxI* gene of yeast conferred salt tolerance in *Arabidopsis*. Transgenic *Arabidopsis* and tomato plants that overexpress *AtNHX1* accumulated abundant quantities of the transporter in the tonoplast and exhibited substantially enhanced salt tolerance (Zhang and Blumwald 2001). Transgenic groundnut plants expressing *AtNHX1* gene showed more resistance to high concentration of salt and water deprivation due to higher level of salt and proline (Asif et al. 2011).

It has been observed that transferring a single gene encoding a single specific stress protein may not be sufficient to reach the required tolerance levels (Bohnert et al. 1995). Therefore, use of gene encoding a stress-inducible transcription factor has been suggested as an alternative for enhancing tolerance towards multiple stresses (Yamaguchi-Shinozaki et al. 1994; Chinnusamy et al. 2005). Several studies showed that overexpression of drought-responsive transcription factors can enhance abiotic stress tolerance in plants (see review, Zhang et al. 2004). For example, overexpression of an ethylene response factor controlled by gene *Sub1A* in rice conferred enhanced submergence tolerance (Xu et al. 2006). Manipulating *CBF/DREB* genes confer improved drought tolerance in crop plants. Transgenic tomato plants expressing *CBF1*, containing three copies of an ABA-responsive complex (ABRC1) from the barley *HAV22* gene, exhibited enhanced tolerance to chilling, water deficit and salt stress (Lee et al. 2003). In another study, expression of *CBF/DREB* genes under stress-inducible promoters in transgenic plants is reported to minimise the growth retardation and other adverse effects (Al-Abed et al. 2007). Development of transgenic with the use of a single regulatory gene (*DREB1A* transcription factor) in groundnut regulated the expression of downstream genes leading

to the activation of many functional genes (Bhatnagar-Mathur et al. 2009). Another transcription factor that has been manipulated in order to increase plant drought tolerance is the *HARDY* (*HRD*) gene, which has been linked to increased transpiration efficiency related to stomatal adjustment. HRD is an AP2/ERF-like transcription factor isolated from hrd-dominant (*hrd-D*) *Arabidopsis* mutants, which displayed vigorous rooting and dark green leaves that were smaller and thicker than WT plants. Karaba et al. (2007) isolated the HRD gene and constitutively expressed it in *Arabidopsis* under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Transgenic rice seedlings, expressing *OsWRKY11* under the control of a rice HSP promoter, HSP101, were shown to survive longer and lose less water under a short and severe drought treatment (Wu et al. 2008). Young transgenic rice plants overexpressing *ZFP252* survived longer, displayed less relative electrolyte leakage and accumulated more compatible osmolytes than WT plants during a 14-day period of drought stress (Xu et al. 2008). A salt- and drought-induced RING-finger protein, SDIR1, was found to confer enhanced drought tolerance to tobacco and rice (Zhang et al. 2008).

Prior to transcriptional activation of genes, drought stress signals are received and messages conveyed to the appropriate components of the downstream pathway (Xiong and Ishitani 2006). Receptor molecules that have been identified to date in plants include ROP10, a small G protein from the ROP family of Rho GTPases, that negatively regulates ABA response in *Arabidopsis* (Zheng et al. 2002); ATHK1, a putative homolog of the yeast SLN1, which is a functional histidine kinase feeding into the HOG MAPK pathway (Reiser et al. 2003); NtC7, a receptor-like membrane protein from tobacco (Tamura et al. 2003) and Cre1, a putative cytokinin sensor and histidine kinase from Arabidopsis (Reiser et al. 2003). The *ERECTA* gene from *Arabidopsis* is the first gene to be shown to act on the coordination between transpiration and photosynthesis (Masle et al. 2005).

Few known studies have focused on engineering heavy metal tolerance in plants. For example, Zhang et al. (2008) reported an aquaporin gene *BjPIP1* from the heavy metal hyperaccumulator Indian mustard, which is upregulated in leaves under drought, salt, low temperature and heavy metal stress. Constitutive expression of *BjPIP1* in tobacco decreased water loss rate, transpiration rate and stomatal conductance of transgenic plants compared to WT under osmotic stress.

5.6.2 Tolerance to Biotic Stresses

Plants sense and respond to environmental cues by a repertoire of mechanisms that regulate gene expression in order to maximise chances of survival in hostile environments (Dorantes-Acosta et al. 2012). In addition to preformed defence traits, plants have evolved inducible defences against microbial pathogens, herbivores and even other plants that involve the regulation of gene expression for the synthesis of defensive secondary metabolites and specific proteins (Walling 2000; Mithofer and Boland 2012).

5.6.2.1 Insect Resistance

Bt technology has emerged as a powerful modality for battling some of the important insect pests. It is a chemical free and economically viable approach for insect pest control in plants (Gatehouse 2008; Pratap and Gupta 2009; de Villiers and Hoisington 2011). Transgenic Bt crops are used worldwide to control major pests (caterpillars and rootworms) of cotton, corn and soybean. The first widely planted Bt crop cultivars were corn producing Bt toxin Cry1Ab and cotton producing Bt toxin *Cry1Ac* (Tabashnik et al. 2009). However, resistance in five lepidopteran pests against the Bt crops carrying the different genes (*Cry1Ab, Cry1F, Cry1Ac* and *Cry2Ab*) has also been reported in South Africa, Puerto Rico, India, the USA and Australia (Kruger et al. 2009; Tabashnik et al. 2008, 2009; Downes et al. 2010). In spite of this, the area under the Bt crops has been increasing since 1996 and in 2011, and biotech crops were grown on 160 million hectares (James 2011). The USA had the largest share of global biotech crop plantings in 2011 (69 million ha), followed by Brazil (30.3 M ha). The other main countries planting biotech crops in 2011 were Argentina (23.7 M ha), India (10.6 M ha) and Canada (10.4 M ha). Brookes and Barfoot (2005) reported that 725 approvals for commercial cultivation had been granted for 155 events in 24 crops, and 57 countries globally have granted regulatory approvals for biotech crops for import for food and feed use and for release into the environment since 1996 (Karthikeyan et al. 2012).

5.6.2.2 Virus Resistance

Plant viruses constitute one of the major problems of the agricultural production worldwide. To date, there are no therapeutical measures available for the control of plant-virus diseases in the field, and the main control strategy used in practice is based on prevention measures. Host plant resistance is by far the most effective way to control plant viruses. However, 'traditional' genetic sources of resistance to viruses are rare, and due to the high rate of mutation of the viral genomes this resistance even when applicable is frequently broken under field conditions. *Agrobacterium*-mediated genetic transformation technology (Thomashow et al. 1980) offered new promising prospects for engineered genetic resistance to viruses with numerous following studies reporting a successful use of the transgenic technology against almost all genera of plant viruses or even viroids (Prins et al. 2008, Schwind et al. 2009). The breakthrough for the creation of transgenic resistance to plant viruses came by Beachy's group which showed that the expression of the coat protein gene of tobacco mosaic virus (TMV) in transgenic plants confers resistance to TMV (Abel et al. 1986).

RNA silencing-based resistance against viruses was first reported by Lindbo et al. (1993) and was shown to be related to the previously observed co-suppression mechanism (Van der Krol et al. 1990). It has been reported that short genome incomplete sequences can be used, and efficiencies of up to 90 % of all transgenic plants produced to be resistant to the homologous virus were achieved (Lin et al.

2007; Tenllado et al. 2004). In order to overcome the weakness of RNA silencing-based resistance Bucher et al. (2006) fused 150-nt fragments of viral sequences of four tospoviruses in a single small chimeric IR construct. This strategy resulted in a high frequency of resistant plants. A most recent approach used modified plant miRNA cistrons to produce a range of antiviral artificial miRNAs (amiRNAs) (Niu et al. 2006; Qu et al. 2007; Zhang et al. 2011). Vasillakos (2012) reviewed that recent advances like the construction of chimeric IR constructs incorporating sequences derived from different virus species if combined with epidemiological data and pest risk analyses could reduce the effect of mixed virus infections on the resistance (Dafny-Yelin and Tzfira 2007; Kung et al. 2009). Recently, virus resistance was achieved through the expression of amiRNAS against viral coding sequences (Ding and Voinnet 2007; Duan et al. 2008; Zhang et al. 2011). Although there was evidence that amiRNA-mediated virus resistance may not be inhibited by low temperature (Niu et al. 2006) this possibly depends on the plant species examined (Qu et al. 2007). Moreover, the durability of this approach, which resulted in relatively few antiviral small RNAs compared with those of the long dsRNA approach, needs to be further demonstrated (Duan et al. 2008; Simon-Mateo and Antonio Garcia 2006).

5.6.2.3 Fungus and Bacteria Resistance

Significant yield losses due to fungal attacks occur in most of the agricultural and horticultural species. In Indian context, fungal diseases are rated either the most important or second most important factor contributing to yield losses in our major cereals, pulses and oilseed crops. The most significant development in the area of varietal development for disease resistance is the use of the techniques of gene isolation and genetic transformation to develop transgenics resistant to fungal diseases.

Genetic engineering allows for introduction of resistance genes from unrelated plant species, which often remain functional in the new host plant (Collinge et al. 2008). The R-gene *Rxo1* from maize was successfully introduced into rice and conferred resistance against bacterial streak disease caused by *Xanthomonas oryzae* pv. *oryzicola* (Zhao et al. 2005). Additional examples of this strategy involve the R-gene *RCT1* from *Medicago truncatula* that was expressed in alfalfa and conferred resistance to *Colletotrichum trifolii* (Yang et al. 1996) and *RPI-BLB2* from wild potato, *Solanum bulbocastanum,* conferring resistance to *Phytophthora infestans* in cultivated potato (Van der Vossen et al. 2005). Chitinase (PR) originating from mycoparasitic biocontrol agents, most notably *Trichoderma harzianum*, that can exhibit higher anti-fungal activity than plant chitinases, has been proven to be a more effective source for enhancing fungal disease resistance in transgenic plants (Dana et al. 2006; Kumar et al. 2009).

In contrast to biotrophic pathogens, necrotrophs produce copious amounts of pathogenicity factors, including toxins and cell wall-degrading enzymes, as a means of successfully establishing infections. Mutants lacking these pathogenicity factors often have reduced virulence or in some instances are completely avirulent.

Overexpression of PGIPs in transgenic plants has successfully reduced disease symptoms due to *B. cinerea* (Joubert et al. 2007; Manfredini et al. 2005) and *Bipolaris sorokiniana* (Janni et al. 2008). Similarly, antisense suppression of PGIPs in *Arabidopsis* increased susceptibility towards *B. cinerea* (Ferrari et al. 2006). The main strategy to resist *Sclerotinia sclerotiorum* infection includes wheat oxalate oxidase and oxalate decarboxylase, converting oxalic acid to CO2 and hydrogen peroxide or CO_2 and formate, respectively. Overexpression of these enzymes in lettuce (Dias et al. 2006), sunflower (Hu et al. 2003), soybean (Cober et al. 2003), rapeseed (Dong et al. 2008) and tomato (Walz et al. 2008) demonstrated at least partial resistance to *S. sclerotiorum*. Adaptation of these technologies will only progress once the costs associated with growing, developing and registering the transgenic technologies are balanced by the gains observed by the producers and ultimately with the consumers of the plants. Once the economic threshold is passed and the plants can be proven safe to be consumed, large-scale adoption of these technologies may become a reality (Wally and Punja 2010).

5.7 Regulations and Biosafety Concerns

Biosafety issue has already become a crucial factor in constraining the further development of transgenic biotechnology and wider application of GM products in agriculture. The most important concerns can be summarized as follows: (1) direct and indirect effects of toxic transgenes (e.g. the Bt insect-resistance gene) to non-target organisms (O'Callaghan et al. 2005; Oliveira et al. 2007); (2) influences of transgenes and GM plants on biodiversity, ecosystem functions and soil microbes (Giovannetti et al. 2005; Oliveira et al. 2007); (3) transgene escape to crop landraces and wild relatives through gene flow and its potential ecological consequences (Mercer et al. 2007) and (4) potential risks associated with the development of resistance to biotic-resistance transgenes in the target organisms (Wu 2007). Among the above environmental biosafety issues, transgene escape from a GM crop variety to its non-GM crop counterparts or wild relatives has aroused tremendous debates worldwide (Ellstrand 2001, 2003). Transgene escape may result in potential ecological consequences if significant amounts of transgenes constantly move to non-GM crops and wild relative species.

The development of marker-free transgenic plants could solve the issues of biological and biosafety in the genetically engineered (GE) crops, besides supporting multiple transformation cycles for transgene pyramiding (Vaucheret et al. 1998). The presence of SMG is undesirable as per the European regulatory agencies' norms. Also trasngene integration at random positions in the genome leads to possible unwanted side effects (mutation) and unpredictable expression patterns. In addition to the risk of HGT, there is also a 'vertical cross-species' transfer risk that could potentially create enhanced weediness problems (Dale et al. 2002). The production of marker-free transgenic crops eliminates the risk of HGT and could mitigate vertical gene transfer. In view of the biosafety requirements, it is recommended

to phase out the SMG since these are unnecessary once an intact transgenic plant has been identified and established (Darbani et al. 2007). Besides, there are public concerns about the widespread occurrence of SMG in novel ecosystems as these are integrated into the plant genome along with the gene of interest (Daniell et al. 2001). Transfer of plant DNA into microbial or mammalian cells under normal conditions of dietary exposure would require all of the following events to occur: (1) removal of the relevant gene(s) from the plant genome, probably as linear fragments; (2) protection of the gene(s) from nuclease degradation in the plant as well as animal gastrointestinal tract; (3) uptake of the gene(s) with dietary DNA; (4) transformation of bacteria or competent mammalian cells; (5) insertion of the gene(s) into the host DNA by rare repair or recombination events into a transcribable unit and finally (6) continuous stabilization of the inserted gene (FAO/WHO 2000; Tuteja et al. 2012).

5.8 Conclusions and Future Prospects

Genetic transformation of crop plants has emerged as a remarkable achievement in modern biotechnology. Transgenic plant varieties hold great promise for augmenting agricultural production and productivity when properly integrated into traditional agricultural research systems. From the recent advances in genetics and genomics it is clear that gene transfer is emerging as a major player in the understanding of gene function and its validation and also that it has a potential to play an important role in generating genetic novelties that, once traits are introduced in the field, should find their way into the breeding strategies for a number of crops. Owing to the utility of this technique, the use of transgenic crop varieties having resistance to a wider range of biotic and abiotic stresses is expected to gain more popularity. However, at the same time, the concerns of general public regarding the safety issues as well as their impact on environment need to be properly addressed. Advancements in removal of selectable markers from the transgenics once they are identified and detailed and unbiased studies on transgene escape to the environments and their real ecological impact may help to a great extent in tackling public scepticism about the development and use of transgenics.

References

Abel PP, Nelson RS, De B, Hoffmann N, Rogers SG, Fraley RT, Beachy RN (1986) Delay of disease development in transgenic plants that express the Tobacco mosaic virus coat protein gene. Science 232:738–743

Al-Abed D, Madasamy P, Talla R, Goldman S, Rudrabhatla S (2007) Genetic engineering of maize with the Arabidopsis DREB1A/CBF3 gene using split-seed explants. Crop Sci 47:2390–2402

Alimohammadi M, Bagherieh-Najjar MB (2009) *Agrobacterium*-mediated transformation of plants: Basic principles and influencing factors. Afr J Biotechnol 8:5142–5148

Altpeter F, Vasil V, Srivastava V, Stöger E, Vasil IK (1996) Accelerated production of transgenic wheat (*Triticum aestivum* L.) plants. Plant Cell Rep 1:612–617

Araki H, Jearnpipatkul A, Tatsumi H, Sakurai T, Ushino K, Muta T, Oshima Y (1987) Molecular and functional organization of yeast plasmid pSR1. J Mol Biol 182:191–203

Arencibia AD, Carmona ERC, Tellez P, Chan MT, Yu SM, Trujillo LE, Oramas P (1998) An efficient protocol for sugarcane (*Saccharum s*:L) transformation mediated by *Agrobacterium tumefaciens*. Transgenic Res 7:213–222

Armaleo D, Ye GN, Klein TM, Shark KB, Sanford JC, Johnston SA (1990) Biolistic nuclear transformation of *Saccharomyces cerevisiae* and other fungi. Curr Genet 17:97–103

Armstrong CL, Rout JR (2001) A novel Agrobacterium-mediated plant transformation method. Int Patent Publ WOO1/09302 A2

Asif MA, Zafar Y, Iqbal J, Iqbal MM, Rashid U, Ali GM, Arif A, Nazir F (2011) Enhanced expression of AtNHX1, in transgenic groundnut *Arachis hypogaea* L. improves salt and drought tolerance. Mol Biotechnol 49:250–256

Bai X, Wang Q, Chu C (2008) Excision of a selective marker in transgenic rice using a novel Cre/loxP system controlled by a floral specific promoter. Transgenic Res 17:1035–1043

Barampuram S, Zhang ZJ (2011) Recent advances in plant transformation. Methods Mol Biol 701:1–35

Barghchi M (1995) High-Frequency and efficient *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* ecotypes "24" and "Landsberg erecta" using *Agrobacterium tumefaciencs*. In: Gartland KMA, Davey MR (eds) Methods in molecular biology: *Agrobacterium* protocols, vol 44. Humana Press Inc, Totowa, NJ, pp 135–147

Barro F, Cannell ME, Lazzeri PA, Barcelo P (1998) The influence of auxins on transformation of wheat and tritordeum and analysis of transgene integration patterns in transformants. Transgenic Res 97:684–695

Bates GW (1994) Genetic transformation of plants by protoplast electroporation. Mol Biotechnol 2:135–145

Beclin C, Charlot F, Botton E, Jouanin L, Dore C (1993) Potential use of aux2 gene from *Agrobacterium* rhizogenes as a conditional negative marker in transgenic cabbage. Transgenic Res 2:48–55

Bhatnagar M, Prasad K, Bhatnagar-Mathur P, Narasu ML, Waliyar F, Sharma KK (2010) An efficient method for the production of marker-free transgenic plants of peanut (*Arachis hypogaea* L.). Plant Cell Rep 29:495–502

Bhatnagar-Mathur P, Vadez V, Devi MJ, Lavanya M, Vani G, Sharma KK (2009) Genetic engineering of chickpea *Cicer arietinum* L. with the P5CSF129A gene for osmoregulation with implications on drought tolerance. Mol Breed 23:591–606

Bidney D, Scelonge C, Martich J, Burrus M, Sims L, Huffman G (1992) Microprojectile bombardment of plant tissues increase transformation frequency by *Agrobacterium* tumefaciens. Plant Mol Biol 18:301–313

Bilang R, Zhang S, Leduc N, Iglesias VA, Gisel A, Simmonds J, Potrykus I, Sautter C (1993) Transient gene expression in vegetative shoot apical meristems of wheat after ballistic micro-targeting. Plant J 4:735–744

Birch RG (1997) Plant transformation problems and strategies for practical application. Annu Rev Plant Physiol Plant Mol Biol 48:297–326

Bohnert HJ, Nelson DF, Jenson RG (1995) Adaptation to environmental stresses. Plant Cell 7:1099–1111

Bolton GW, Nester EW, Gordon MP (1986) Plant phenolic compounds induce expression of the *Agrobacterium tumefaciens* loci needed for virulence. Science 232:983–985

Borda's M, Montesinos C, Dabauza M, Salvador A, Roig LA, Serrano R, Moreno V (1997) Transfer of the yeast salt tolerance gene HAL1 to *Cucumis melo* L. cultivars and in vitro evaluation of salt tolerance. Transgenic Res 5:1–10

Bottinger P, Steinmetz A, Scheider O, Pickardt T (2001) *Agrobacterium* mediated transformation of *Vicia faba*. Mol Breed 8:243–254

Bray EA (1993) Molecular responses to water deficit. Plant Physiol 103:1035–1040

Brookes G, Barfoot P (2005) GM crops The global economic and environmental impact. The first nine years 1996–2004. AgBioForum 8:187–196

Bucher E, Lohuis D, van Popple PMJA, Geerts-Dimitriadou C, Goldbach R, Prins M (2006) Multiple virus resistance at a high frequency using a single transgene construct. Journal of General Virology 87:3697–3701

Cadoza V, Stewart CN (2003) Increased *Agrobacterium* mediated transformation and rooting efficiencies in canola (*Brassica napus* L.) from hypocotyls segment explants. Plant Cell Rep 21:599–604

Capell T, Bassie L, Christou P (2004) Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. Proc Natl Acad Sci USA 101:9909–9914

Castillo AM, Vasil V, Vasil IK (1994) Rapid production of fertile transgenic plants of rye *Secale cereale* L. Bio Technol 12:1366–1371

Chang SS, Park SK, Kim BC, Kang BJ, Kim DU (1994) Stable genetic transformation of Arabidopsis thaliana by *Agrobacterium* inoculation in planta. Plant J 5:551–558

Chee PP, Fober KA, Slightom JL (1989) Transformation of soybean (Glycine max) by *Agrobacterium tumefaciens*. Plant Physiol 91:1212–1218

Cheng M, Hu T, Layton JI, Liu CN, Fry JE (2003) Desiccation of plant tissues post-Agrobacterium infection enhances T-DNA delivery and increases stable transformation efficiency in wheat. In Vitro Cell Dev Biol Plant 39:595–604

Cheng M, Fry JE (2000) An improved efficient Agrobacterium-mediated plant transformation method. Int. Patent publ. WO 0034/491

Cheng M, Fry JE, Pang S, Zhou I, Hironaka C, Duncan DRI, Conner TWL, Wang Y (1997) Genetic transformation of wheat mediated by *Agrobacterium* tumefaciens. Plant Physiol 115:971–980

Chibbar RN, Kartha KK, Leung N, Qureshi J, Caswell K (1991) Transient expression of marker genes in immature zygotic embryos of spring wheat (*Triticum aestivum*) through microparticle bombardment. Genome 34:453–460

Chinnusamy V, Jagendorf A, Zhu JK (2005) Understanding and improving salt tolerance in plants. Crop Sci 45:437–448

Cho YG (2009) Auto-excision of selectable marker genes from transgenic tobacco via a stress inducible FLP/FRT site-specific recombination system. Transgenic Res 18:455–465

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY (1995) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sip 18:659–668

Clough S, Bent JAF, Dip F (1998) A simplified method for *Agrobacterium* mediated transformation of Arabidopsis thaliana. Plant J 16:735

Cober ER, Rioux S, Rajcan I, Donaldson PA, Simmonds DH (2003) Partial resistance to white mold in a transgenic soybean line. Crop Sci 4:392–395

Collinge DB, Lund OS, Thordal-Christensen H (2008) What are the prospects for genetically engineered, disease resistant plants? Eur J Plant Pathol 121:217–231

Cortina C, Culianez-Macia F (2005) Tomato abiotic stress enhanced tolerance by trehalose biosynthesis. Plant Sci 16:75–82

Cuellar W, Gaudin A, Solorzano D, Casas A, Nopo L, Chudalayandi P, Medrano G, Kreuze J, Ghislain M (2006) Self-excision of the antibiotic resistance gene nptII using a heat inducible Cre-loxP system from transgenic potato. Plant Mol Biol 62:71–82

Cui M, Takayanagi K, Kamada H, Nishimura S, Handa T (2001) Efficient shoot regeneration from hairy roots of Antirrhinum majus L transformed by the rol-type MAT vector system. Plant Cell Rep 20:55–59

Czako M, Marton L (1994) The herpes simplex virus thymidine kinase gene as a conditional negative-selection marker gene in *Arabidopsis thaliana*. Plant Physiol 104:1067–1071

Dafny-Yelin M, Tzfira T (2007) Delivery of multiple transgenes to plant cells. Plant Physiol 145:1118–1128

Dale PJ, Irwin JA, Scheffler JA (1993) The experimental and commercial release of transgenic crop plants. Plant Breed 111:1–22

Dale EC, David WO (1991) Gene transfer with subsequent removal of the selection gene from the host genome. Proc Natl Acad Sci USA 88:10558–10562

Dale PJ, Clarke B, Fontes EMG (2002) Potential for the environmental impact of transgenic crops. Nat Biotechnol 20:567–574

Dana MD, Pintor-Toro JA, Cubero B (2006) Transgenic tobacco plants overexpressing chitinases of fungal origin show enhanced resistance to biotic and abiotic stress agents. Plant Physiol 142:722–730

Daniell H, Wiebe PO, Millan AF (2001) Antibiotic-free chloroplast genetic engineering—an environmentally friendly approach. Trend Plant Sci 6:237–239

Darbani B, Elimanifar A, Stewart CN, Camargo WN (2007) Methods to produce marker-free transgenic plants. Biotechnol J 2:83–90

De Block M and Debrouwer D (1991) Two T-DNA's cotransformed into *Brassica napus* by a double *Agrobacterium tumefaciens* infection are mainly integrated at the same locus. Theor Appl Genet 82:257–263

de Neve M, de Buck S, Jacobs A, van Montagu M, Depicker A (1997) T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. Plant J 1:15–29

de Villiers SM, Hoisington AD (2011) The trends and future of biotechnology crops for insect pest control. Afr J Biotechnol 10:4677–4681

Dehestani A, Ahmadian G, Salmanian AH, Jelodar NB, Kazemitabar K (2010) Transformation efficiency enhancement of *Arabidopsis* vacuum infiltration by surfactant application and apical inflorescence removal. Trakia J Sci 81:19–26

Depicker AG, Jacobs AM, van Montagu MC (1988) A negative selection scheme for tobacco protoplast-derived cells expressing the T-DNA gene. Plant Cell Rep 7:63–66

Desfeux C, Clough SJ, Bent AF (2000) Female reproductive tissues are the primary target of *Agrobacterium*-mediated transformation by the Arabidopsis floral-dip method. Plant Physiol 12:895–904

Dias BBA, Cunha WG, Morais LS, Vianna GR, Rech EL, de Capdeville G et al (2006) Expression of an oxalate decarboxylase gene from *Flammulina sp* in transgenic lettuce (*Lactuca sativa)* plants and resistance to *Sclerotinia sclerotiorum*. Plant Pathol 55:187–193

Dillen W, de Clercq J, Kapila J, Zambre M, van Montagu M, Angenon G (1997) The effect of temperature on *Agrobacterium* tumefaciens-mediated gene transfer to plants. Plant J 12: 1459–1463

Ding SW, Voinnet O (2007) Antiviral immunity directed by small RNAs. Cell 130:413–426

Dong J, Teng W, Buchholz WG, Hall TC (1996) *Agrobacterium*-mediated transformation of Javanic rice. Mol Breeding 2:267–276

Dong XB, Ji RQ, Guo XL, Foster SJ, Chen H, Dong CH et al (2008) Expressing a gene encoding wheat oxalate oxidase enhances resistance to *Sclerotinia sclerotiorum* in oilseed rape (*Brassica napus*). Planta 228:331–340

Dorantes-Acosta AE, Sánchez-Hernández CV, Arteaga-Vázquez MA (2012) Biotic stress in plants life lessons from your parents and grandparents. Front Gene 32:56

Downes S, Mahon RJ, Rossiter L, Kauter G, Leven T, Fitt G, Baker G (2010) Adaptive management of pest resistance by Helicoverpa species (Noctuidae) in Australia to the Cry2Ab Bt toxin in Bollgard II ® cotton. Evol Appl 3:574–584

Draper J, Scott R, Armitage P (1988) Plant genetic transformation and gene expression a laboratory manual. Blackwell Scientific Publishers, Oxford

Duan CG, Wang CH, Fang RX, Guo HS (2008) Artificial MicroRNAs highly accessible to targets confer efficient virus resistance in plants. J Virol 82:11084–11095

Ebinuma H, Sugita K, Matsunaga E, Yamakado M (1997) Selection of marker-free transgenic plants using the isopentenyl transferase gene. Proc Natl Acad Sci USA 94:2117–2121

Ebinuma H, Komamine A (2001) MAT multi-auto-transformation vector system. The oncogenes of Agrobacterium as positive markers for regeneration and selection of marker-free transgenic plants. In Vitro Cell Dev Biol Plant 37:103–113

Ellstrand NC (2001) When transgeneswander shouldweworry? Plant Physiol 125:1543–1545
Ellstrand NC (2003) Current knowledge of gene flow in plants: implications for transgene flow. Philos Trans R Soc B Biol Sci 35:1163–1170
Enriquez-Obregon GA, Prieto-Samsonov DL, de la Riva GA, Perez MI, Selman-Housein G, Vazquz-Padron RI (1999) *Agrobacterium*mediated Japonica rice transformation a procedure assisted by an antinecrotic treatment. Plant Cell Tiss Organ Cult 59:159–168
Feldmann KA, Marks MD (1987) *Agrobacterium*-mediated transformation of germinating seeds of Arabidopsis thaliana a non-tissue culture approach. Mol Gen Genet 20:81–89
Ferrari S, Galletti R, Vairo D, Cervone F, de Lorenzo G (2006) Antisense expression of the Arabidopsis thaliana AtPGIP1 gene reduces polygalacturonase-inhibiting protein accumulation and enhances susceptibility to *Botrytis cinerea*. Mol Plant Microbe Interact 19:931–936
Fladung M, Schenk TMH, Polak O, Becker D (2010) Elimination of marker genes and targeted integration via FLP/FRT recombination system from yeast in hybrid aspen Populus tremula L P tremuloides Michx. Tree Genet Genome 6:205–217
Food and Agriculture Organization/World Health Organization (2000) Safety aspects of genetically modified foods of plantorigin. Report of a Joint FAO/WHO Consultation on FoodsDerived from Biotechnology (Geneva: World Health Organization)
Frame BR, Shou H, Chikwamba RK, Zhang ZI, Xiang CI, Fonger TM, Pegg SEK, Li B, Nettleton DS, Pei D, Wang K (2002) *Agrobacterium* tumefaciens-mediated transformation of maize embryos using a standard binary vector system. Plant Physiol 129:13–22
Fry J, Barnason A, Horsch RB (1987) Transformation of Brassica napus with *Agrobacterium* tumefaciens based vectors. Plant Cell Rep 6:321–325
Fu X, Duc LT, Fontana S, Bong BB, Tinjuangjun P, Sudhakar D, Twyman RM, Christou P, Kohli A (2000) Linear transgene constructs lacking vector backbone sequences generate low-copynumber transgenic plants with simpl integration patterns. Transgenic Res 9:11–19
Galau GA, Bijaisoradat N, Hughes DW (1987) Accumulation kinetics of cotton late embryogenesis-abundent (Lea) mRNAs and storage protein mRNAs coordinate regulation during embryogenesis and role of abscisic acid. Dev Biol 123:198–212
Gan C (1989) Gene gun accelerates DNA-coated particles to transform intact cells. The Scientist 3:25
Gao M, Sakamoto A, Miura K, Murata N, Sugiura A, Tao R (2000) Transformation of Japanese persimmon (*Diospyros kaki* Thunb.) with a bacterial gene for choline oxidase. Mol Breed 6:501–510
Gao Z, Xie X, Ling Y, Muthukrishnan S, Liang HG (2005) *Agrobacterium* tumefaciens transformation using a mannose selection system. Plant Biotechnol J 3:591–597
Garg AK, Kim JK, Owens TG, Ranwala AP, Choi YC, Kochian LV, Wu RJ (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci USA 99:15898–15903
Gatehouse JA (2008) Biotechnological prospects for engineering insect-resistant plants. Plant Physiol 146:881–887
Gelvin SB (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annu Rev Plant Physiol Plant Mol Biol 51:223–256
Gheysen G, Angenon G, van Montagu M (1998) *Agrobacterium*-mediated plant transformation a scientifically intriguing story with significant applications. In: Lindsey K (ed) Transgenic Plant Research. Harwood Academic Publishers, New Jersey, NJ, pp 1–33
Giovannetti M, Sbrana C, Turrini A (2005) The impact of genetically modified crops on soil microbial communities. Riv Biol-Biol Forum 98:393–417
Gisbert C, Rus AM, Bolarin MC, Lopez-Coronado M, Arrillaga I, Montesinos C, Caro M, Serrano R, Moreno V (2000) The yeast HAL1 gene improves salt tolerance of transgenic tomato. Plant Physiol 123:393–402
Gleave AP, Mitra DS, Mudge S, Morris BAM (1999) Selectable marker-free transgenic plants without sexual crossing transient expression of *Cre* recombinase and use of the conditional lethal dominant gene. Plant Mol Biol 40:223–235

Godwin I, Todd G, Ford-Lloyd B, Newbury HJ (1991) The effects of acetosyringone and pH on *Agrobacterium* mediated transformation vary according to plant species. Plant Cell Rep 9:671–675

Goldsbrough AP, Lastrella CN, Yoder JI (1993) Transposition-mediated re-positioning and subsequent elimination of marker genes from transgenic tomatoes. Biotechnology 11:1286–1292

Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O´brien JV, Chambers SA, Adams WR, Jr Willetts NG, Rice TB, Mackey CJ, Krueger RW, Kausch AP, Lemaux PG (1990) Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2:603–618

Guo M, Zhang YL, Meng ZJ, Jiang J (2012) Optimization of factors affecting *Agrobacterium-mediated* transformation of Micro-Tom tomatoes. Genet Mol Res 111:661–671

Haldrup A, Petersen SG, Okkels FT (1998) Positive selection: a plant selection principle based on xylose isom-erase an enzyme used in the food industry. Plant Cell Rep 18:76–81

Hansen G, Chilton MD (1996) 'Agrolistic' transformation of plant cells integration of T-strands generated in planta. Proc Natl Acad Sci USA 93:14978–14983

Hashizume F, Tsuchiya T, Ugaki M, Niwa Y, Tachibana N, Kowyama Y (1999) Efficient *Agrobacterium*-mediated transformation and the usefulness of a synthetic GFP reporter gene in leading varieties of japonical rice. Plant Biotechnol 16:397–401

Herrera-Estrella L, De Block M, Messens E, Heradsteens J, van Montagu M and Schell J (1983) Chimeric genes as dominant selectable markers in plant cells. EMBO J 2:987–995

Hiei Y, Komari T, Kubo T (1997) Transformation of rice mediated by *Agrobacterium tumefaciens*. Plant Mol Biol 35:205–218

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oriza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282

Hoa TT, Bong BB, Huq E, Hodges TK (2002) Cre/lox sitespecific recombination controls the excision of a transgene from the rice genome. Theor Appl Genet 104:518–525

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperpoort RA (1983) A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium* t*umefaciens* Ti-plasmid. Nature 303:179–180

Hood EE, Helmer GL, Fraley RT, Chilton MD (1986) The hypervirulence of *Agrobacterium* tumefaciens A281 is encoded in a region of pTi Bo542 outside of T-DNA. J Bacteriol 168:1291–1301

Howe AR, Gasser CS, Brown SM, Padgette SR, Hart J, Parker G, Fromm ME, Armstrong CL (2002) Glyphosate as a selective agent for production fertile transgenic maize (*Zea mays L.*) plant. Mol Breed 10:153–164

Hu T, Meltz S, Chay C, Zhou HP, Biest N, Chen G, Cheng M, Feng X, Radionenka M, Lu F, Fry JE (2003) *Agrobacterium*-mediated large scale transformation of wheat (*Triticum aestivum* L.). Plant Cell Rep 21:1010–1019

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nat Biotechnol 14:745–750

Ishitani M, Xiong L, Stevenson B, Zhu JK (1997) Genetic analysis of osmotic and cold stress signal transduction in Arabidopsis interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. Plant Cell 9:1935–1949

James C (2011) Global Status of Commercialized Biotech/GM Crops, vol 43. ISAAA Brief, Ithaca, USA

Janni M, Sella L, Favaron F, Blechl AE, De Lorenzo G, D'Ovidio R (2008) The expression of a bean PGIP in transgenic wheat confers increased resistance to the fungal pathogen Bipolaris sorokiniana. Mol Plant Microbe Interact 21:171–177

Jefferson RA, Kavanagh TA, Beven MW (1987) GUS fusions bglucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Joersbo M, Okkels FT (1996) A novel principle for selection of transgenic plant cells: positive selection. Plant Cell Rep 16:219–221

Joersbo M, Brunstedt J (1990) Direct gene transfer to plant protoplasts by mild sonication. Plant Cell Rep 9:207–210

Joersbo M, Donaldson I, Kreiber J, Peterson SG, Brunstedt J, Okkels FT (1998) Analysis of mannose selection used for transformation of sugar beet. Mol Breed 4:111–117

Joubert DA, Kars I, Wagemakers L, Bergmann C, Kemp G, Vivier MA, van Kan JAL (2007) A polygalacturonase-inhibiting protein from grapevine reduces the symptoms of the endopolygalacturonase BcPG2 from Botrytis cinereain Nicotiana benthamiana leaves without any evidence for in vitro interaction. Mol Plant Microbe Interact 20:392–402

Karaba A, Dixit S, Greco R, Aharoni A, Trijatmiko KR, Marsch-Martinez N, Krishnan A, Nataraja KN, Udayakumar M, Pereira A (2007) Improvement of water use efficiency in rice by expression of HARDY, an *Arabidopsis* drought and salt tolerance gene. Proc Natl Acad Sci USA 104:15270–15275

Karlin-Neumann GA, Brusslan JA, Tobin EM (1991) Phytochrome control of the tms2 gene in transgenic Arabidopsis: a strategy for selecting mutants in the signal transduction pathway. Plant Cell 3:573–582

Karthikeyan A, Valarmathi R, Nandini S, Nandhakumar MR (2012) Genetically modified crops insect resistance. Biotechnology 11(3):119–126

Katavic V, Haughn GW, Reed D, Martin M, Kunst L (1994) In planta transformation of *Arabidopsis thaliana*. Mol Gen Genet 2:363–370

Ke XY, McCormac AC, Harvey A, Lonsdale D, Chen DF, Elliot MC (2002) Manipulation of discriminatory T-DNA delivery by *Agrobacterium* into cells of immature embryos of barley and wheat. Euphytica 126:333–343

Keshamma ES, Rohini KS, Madhusudhan RB, Kumar MU, Kumar MU (2008) Molecular biology and physiology. J Cotton Sci 12:264–272

Khan RS, Nakamura I, Mii M (2010a) Production and selection of marker-free transgenic plants of Petunia hybrida using sitespecific recombination. Biol Plant 54:265–271

Khan RS, Ntui VO, Chin DP, Nakamura I, Mii M (2010b) Production of marker-free disease-resistant potato using isopentenyl transferase gene as a positive selection marker. Plant Cell Rep 30:587–597

Khan RS, Thirukkumaran G, Nakamura I, Mii M (2010c) Rol root loci gene as a positive selection marker to produce markerfree Petunia hybrida. Plant Cell Tiss Organ Cult 101:279–285

Khanna HK, Daggard GE (2003) *Agrobacterium* tumefaciens-mediated transformation of wheat using a superbinary vector and a polyaminesupplemented regeneration medium. Plant Cell Rep 2:429–436

Klee H, Horsch R, Rogers S (1987) *Agrobacterium*-mediated plant transformation and its further applications to plant biology. Annu Rev Plant Physiol 38:467–486

Klee H (2000) A guide to *Agrobacterium* binary Ti vectors. Trends Plant Sci 5:446–451

Kobayashi T, Hisajima S, Stougaard J, Ichikawa HA (1995) Conditional negative selection for Arabidopsis expressing a bacterial cytosine deaminase gene. Jpn J Genet 70:409–422

Komari T, Hiei Y, Saito Y, Murai N, Kumashiro T (1996) Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant J 10:165–174

Kondo T, Hasegawa H, Suzuki M (2000) Transformation and regeneration of garlic (*Allium sativum* L.) by *Agrobacterium*-mediated gene transfer. Plant Cell Rep 19:989–993

Koprek T, Hänsch R, Nerlich A, Mendel RR, Schulze J (1996) Fertile transgenic barley of different cultivars obtained by adjustment of bombardment conditions to tissue response. Plant Sci 11:979–991

Kruger M, van Rensburg JBJ, van den Berg J (2009) Perspective on the development of stem borer resistance to Bt maize and refuge compliance at the Vaalharts irrigation scheme in South Africa. Crop Prot 28:684–689

Kumar S, Arul L, Talwar D (2010) Generation of marker-free Bt transgenic indica rice and evaluation of its yellow stem borer resistance. J Appl Genet 51:243–257

Kumar V, Parkhi V, Kenerley CM, Rathore KS (2009) Defense-related gene expression and enzyme activities in transgenic cotton plants expressing an endochitinase gene from Trichoderma virens in response to interaction with Rhizoctonia solani. Planta 230:277–291

Kumlehn J, Serazetdinora L, Hensel G, Becker D, Loerz H (2006) Genetic transformation of barley (*Hordeum vulgare* L.) via infection of androgenetic pollen culture with *Agrobacterum tumefaciens*. Plant Biotechnol J 4:251–258

Kung YJ, Bau HJ, Wu YL, Huang CH,Chen TM, Yeh SD(2009) Generation of transgenic papaya with double resistance to Papaya ringspot virus and Papaya leaf-distortion mosaic virus. Phytopathology 99:1312–1320

Kunze I, Ebneth M, Heim U, Geiger M, Sonnewald U, Herbers K (2001) 2-Deoxyglucose resistance: a novel selection marker for plant transformation. Mol Breed 7:221–227

Le VQ, Belles-Isles J, Dusabenyagusani M, Tremblay FM (2001) An improved procedure for production of white pruce (Picea glauca) transgenic plants using Agrobacterium tumefaciens J Exp Bot 52:2089–2095

Lee JT, Prasad V, Yang PT, Wu JF, David Ho TH, Charng YY, Chan MT (2003) Expression of *Arabidopsis CBF1* regulated by an ABA/stress inducible promoter in transgenic tomato confers stress tolerance without affecting yield. Plant Cell Environ 26:1181–1190

Lee LY, Gelvin SB (2008) T-DNABinary vectors and systems. Plant Physiol 146:325–332

Li B, Li N, Duan X, Wei A, Yang A, Zhang J (2010) Generation of marker-free transgenic maize with improved salt tolerance using the FLP/FRT recombination system. J Biotechnol 145:206–213

Li KT, Charng YC (2012) The use of hygromycin phosphotransferase gene *hpt* with an artificial intron to obtain marker-off transgenic plants. Afr J Biotechnol 116:1330–1336

Limanton-Grevet A, Jullien M (2001) *Agrobacterium*-mediated transformation *Asparagus officinalis* L. Molecular and genetic analysis of transgenic plants. Mol Breed 7:141–150

Lin C, Jun F, Xu X, Zhao T, Cheng J, Tu J, Ye G, Shen Z (2008) A built-in strategy for containment of transgenic plants Creation of selectively terminable transgenic rice. PLoS One 31:818

Lin SS, Henriques R, Wu HW, Niu QW, Yeh SD, Chua NH (2007) Strategies and mechanisms of plant virus resistance. Plant Biotechnol Rep 1:125–134

Lindbo JA, Silva-Rosales L, Proebsting WM, Dougherty WG (1993) Induction of a highly specific antiviral state in transgenic plants implications for regulation of gene expression and virus resistance. Plant Cell 5(17):49–59

Lindsey K, Jones MGK (1989) Plant Biotechnology in Agriculture. Open University Press, Milton Keynes, UK

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18:100–127

Liu X, Brost J, Hutcheon C, Guilfoil R, Wilson AK, Leung S, Shewmaker CK, Rooke S, Nguyen T, Kiser J (2012) Transformation of the oilseed crop Camelina sativa by *Agrobacterium-mediated* floral dip and simple large-scale screening of transformants. In Vitro Cellular Develop Biol 485:462–468

Lonsdale D, Ontec S, Cuming A (1990) Transient expression of exogenous DNA in intact, viable wheat embryos following particle bombardment. J Exp Bot 41:1161–1165

Lucca P, Ye X, Potrykus I (2001) Effective selection and regeneration of transgenic rice plants with mannose as selective agent. Mol Breed 7:43–49

Luo K, Duan H, Zhao D, Zheng X, Deng W, Chen Y, Stewart CN, McAvoy R, Jiang X, Wu Y, He A, Pei Y, Li Y (2007) 'GM-gene-deleter': fused loxP-FRT recognition sequences dramatically improve the efficiency of FLP or CRE recombinase on transgene excision from pollen and seed of tobacco plants. Plant Biotechnol J 5:263–274

Ma BG, Duan XY, Niu JX, Ma C, Hao QN, Zhang LX, Zhang HP (2009) Expression of stilbene synthase gene in transgenic tomato using salicylic acid-inducible Cre/loxP recombination system with self-excision of selectable marker. Biotechnol Lett 31:163–169

Manfredini C, Sicilia F, Ferrari S, Pontiggia D, Salvi G, Caprari C, Lorito M, de Lorenzo G (2005) Polygalacturonase-inhibiting protein 2 of *Phaseolus vulgaris* inhibits *BcPG1*, a polygalacturonase of Botrytis cinerea important for pathogenicity, and protects transgenic plants from infection. Physiol Mol Plant Pathol 67:108–115

Masle J, Gilmore SR, Farquhar GD (2005) The ERECTA gene regulates plant transpiration efficiency in Arabidopsis. Nature 4:866–870

May GD, Afza R, Mason HS, Wiecko A, Novak FJ, Arntzen CJ (1995) Generation of transgenic banana (*Musa acuminata*) plants via *Agrobacterium*-mediated transformation. Bio/Technol 13:486–492

McCormac AC, Fowler MR, Chen DF, Elliott MC (2001) Efficient co-transformation of *Nicotiana tabacum* by two independent T-DNAs the effect of T-DNA size and implications for genetic separation. Transgenic Res 10:143–155

McKnight TD, Lillis MT, Simpson RB (1987) Segregation of genes transferred to one plant cell from two separate *Agrobacterium* strains. Plant Mol Biol 8:439–445

McNeil SD, Nuccio ML, Rhodes D, Shachar-Hill Y, Hanson AD (2000) Radiotracer and computer modeling evidence that phosphobase methylation is the main route of choline synthesis in tobacco. Plant Physiol 12:3371–3380

Mercer KL, Andow DA, Wyse DL, Shaw RG (2007) Stress and domestication traits increase the relative fitness of crop-wild hybrids in sunflower. Ecol Lett 10:383–393

Miller M, Tagliani L, Wang N, Berka B, Bidney D (2002) High efficiency transgene segregation in co-transformed maize plants using an *Agrobacterium* tumefaciens 2 T-DNA binary system. Transgenic Res 11:381–396

Mithofer A, Boland W (2012) Plant defense against herbivores: chemical aspects. Annu Rev Plant Biol 63:431–450

Mlynarova L, Conner AJ, Nap JPH (2006) Directed microspore-specific recombination of transgenic alleles to prevent pollen-mediated transmission of transgenes. Plant Biotechnol J 4:445–452

Moloney MM, Walker JM, Sharma KK (1989) An efficient method for *Agrobacterium*-mediated transformation in Brassica napus cotyledon explants. Plant Cell Rep 8:238–242

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–479

Nandadeva YL, Lupi CG, Meyer CS, Devi PS, Potrykus I, Bilang R (1999) Microprojectile mediated transient and integrative transformation of rice embryogenic suspension cells effect of osmotic cell conditioning and of the physical configuration of plasmid DNA. Plant Cell Rep 18:500–504

Nandy S, Srivastava V (2011) Site-specific gene integration in rice genome mediated by the FLP–FRT recombination system. Plant Biotechnol J 9:713–721

Nanjo T, Kobayashi M, Yoshiba Y, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K (1999) Antisense supression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. FEBS Lett 461:205–210

Nanto K, Ebinuma H (2008) Marker-free site-specific integrationplants. Transgenic Res 17:337–344

Nauerby B, Billing K, Wyndaele R (1997) Influence of the antibiotic timentin on plant regeneration compared to carbenicillin and cefotaxime in concentrations suitable for elimination of *Agrobacterium* tumefaciens. Plant Sci 123:169–177

Niu QW, Lin SS, Reyes JL, Chen KC, Wu HW, Yeh SD, Chua NH (2006) Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. Nat Biotechnol 24:1420–1428

O'Keefe DP, Tepperman JM, Dean C, Leto KJ, Erbes DL, Odell JT (1994) Plant expression of a bacterial cyto-chrome P450 that catalyzes activation of a sulfonylurea pro- herbicide. Plant Physiol 105:473–482

O'Callaghan M, Glare TR, Burgess EPJ, Malone LA (2005) Effects of plants genetically modified for insect resistance on non-target organisms. Annu Rev Entomol 50:271–292

Okkels FT, Ward J, Joersb M (1997) Synthesis of cytoki-nin glucuronides for the selection of transgenic plant cells. Phytochemistry 46:801–804

Oliveira AR, Castro TR, Capalbo DMF, Delalibera I (2007) Toxicological evaluation of genetically modified cotton BollgardR and Dipel RWP on the non-target soil mite *Scheloribates praeincisus* Acari: Oribatida. Exp Appl Acarol 41:191–201

Opabode JT (2006) *Agrobacterium*-mediated transformation of plants: emerging factors that influence efficiency. Biotechnol Mol Biol Rev 11:12–20

Perl A, Kless H, Blumenthal A, Galili G, Galun E (1992) Improvement of plant regeneration and GUS expression in scutellar wheat calli by optimization of culture conditions and DNAmicroprojectile delivery procedures. Mol Gen Genet 235:279–284
Petolino JF, Hopkins NL, Kosegi BD, Skokut M (2000) Whisker-mediated transformation of embryogenic callus of maize. Plant Cell Rep 19:781–786
Pilon-Smits EAH, Ebskamp MJM, Paul MJ, Jeuken JW, Weisbeek, Smeekens SCM (1995) Improved performance of transgenic fructan-accumulating tobacco under drought stress. Plant Physiol 107:125–130
Pradhan C, Das AB, Chand PK (2012) *Agrobacterium tumefaciens*-mediated transformation and efficient regeneration of a timber yielding plant *Dalbergia sissoo* Roxb. Physiol Mol Biol Plants. doi:10.1007/s12298-012-0120-z
Pratap A, Gupta SK (2009) Biotechnological interventions in host plant resistance. In: Peshin R, Dhawan AK (eds) Integrated Pest Management: Innovation, Dissemination and Impact. Springer Publishers, Dordrecht, UK, pp 183–207
Prins M, Laimer M, Noris E,Schubert J, Wassenegger M, Tepfer M (2008) Strategies for antiviral resistance in transgenic plants. Molecular Plant Pathology 1:73–83
Qu J, Ye J, Fang R (2007) Artificial microRNA-mediated virus resistance in plants. J Virol 81:6690–6699
Ramana Rao MV, Parameswari C, Sripriya R, Veluthambi K (2011) Transgene stacking and marker elimination in transgenic rice by sequential *Agrobacterium*-mediated co-transformation with the same selectable marker gene. Plant Cell Rep 307:1241–1252
Ramana Rao MV, Veluthambi K (2010) Selectable marker elimination in the T0 generation by *Agrobacterium*-mediated co-transformation involving Mungbean yellow mosaic virus TrAP as a non-conditional negative selectable marker and bar for transient positive selection. Plant Cell Rep 29:473–483
Rao K, Chodisetti B, Mangamoori LN, Giri A (2012) *Agrobacterium*-mediated transformation in *Alpinia galanga* Linn Willd. for enhanced acetoxychavicol acetate production. Appl Biochem Biotechnol 1682:339–437
Rao SK, Rohini VK (1999) *Agrobacterium* mediated transformation of sunflower (*Helianthus annus* L.) a simple protocol. Ann Bot 83:347–354
Rasco-Gaunt S, Riley A, Barcelo P, Lazzeri PA (1999) Analysis of particle bombardment parameters to optimise DNA delivery into wheat tissues. Plant Cell Rep 19(118–127):106
Rashid H, Yokoi S, Toriyama K, Hinata K (1996) Transgenic plant production mediated by *Agrobacterium* in Indica rice. Plant Cell Rep 15:727–730
Reichel C, Mathur J, Eckes P, Langenhemper K, Koncz C, Schell J, Reiss B, Maas C (1996) Enhanced green fluorescence by the expression of an *Aequorea victoria* green fluorescent protein mutant in mono and dicotyledonous plant cells. Proc Natl Acad Sci USA 93: 5888–5893
Reiser V, Raitt D, Saito H (2003) Yeast osmosensor *Sln1* and plant cytokinin receptor *Cre1* respond to changes in turgor pressure. Yeast 20:S169
Reiss B, Sprengel R, Will H (1984) A new sensitive method for quantitative and qualitative assay of neomycin phosphotransferase in crude cell extracts. Gene 30:211
Ritala A, Aspegren K, Kurtén U, Salmenkallio-Marttila M, Mannonen L, Hannus R, Kauppinen V, Teeri TH, Enari TM (1994) Fertile transgenic barley by particle bombardment of immature embryos. Plant Mol Biol 24:317–325
Rohini VK, Rao SK (2000a) Embryo transformation—a practical approach for realizing transgenic plants of safflower (*Carthamus tinctorius* L.). Ann Bot 86:1043–1049
Rohini VK, Rao SK (2000b) Transformation of peanut (*Arachis hypogaea* L.) a non-tissue culture based approach for generating transgenic plants. Plant Sci 150:41–49
Russel JA, Roy MK, Sanford JC (1992) Physical trauma and tungsten toxicity reduce the efficiency of biolistic transformation. Plant Physiol 98:1050–1056
Saelim L, Phansiri S, Suksangpanomrung M, Netrphan S, Narangajavana J (2009) Evaluation of a morphological marker selection and excision system to generate marker-free transgenic cassava plants. Plant Cell Rep 28:445–455

Sakamoto A, Valverde R, Alia, Chen TH, Murata N. (2000) Tranformation of *Arabidopsis* with the codA gene for choline oxidase enhances freezing tolerance of plants. Plant J 22:449–453
Salas MC, Park SH, Srivatanakul M, Smith RH (2001) Temperature influence on stable T-DNA integration in plant cells. Plant Cell Rep 20:701–705
Sanford JC, Klein TM, Wolf ED, Allen N (1987) Delivery of substances into cells and tissues using a particle bombardment process. Particul Sci Technol 5:27–37
Schlaman HRM, Hooykaas PJJ (1997) Effectiveness of the bacterial gene codA encoding cytosine deaminase as a negative selectable marker in *Agrobacterium*-mediated plant transformation. Plant J 11:1377–1385
Schwind N, Zwiebel M, Itaya A, Ding B, Wang MB, Krczal G, Wassenegger M (2009) RNAi-mediated resistance to Potato spindle tuber viroid in transgenic tomato expressing a viroid hairpin RNA construct. Mol Plant Pathol 10:459–469
Shan XY, Shan BL, Zhang JR (2006) Production of marker-free transgenic tobacco plants by Flp/frt recombination system. Chinese J Biotechnol 22:744–749
Sharma KK, Anjaiah V (2000) An efficient method for the production transgenic plants for peanut *Arachis hypogea* L. through *Agrobacterium* tumefaciens mediated genetic transformation. Plant Sci 1:597–19
Sharma KK, Bhatnagar-Mathur P, Thorpe TA (2005a) Genetic transformation technology: status and problems. In Vitro Cell Dev Biol Plant 41:102–112
Sharma KK, Bhatnagar-Mathur P, Thorpe TA (2005b) Genetic transformation technology: status and problems. In Vitro Cell Dev Biol 41:102–112
Sharma KK, Bhojwani SS, Thorpe TA (1990) High frequency regeneration of shoots and roots from cotyledon explants of *Brassica juncea* L. Czern Plant Sci 66:247–253
Sheen J, Hwang S, Niwa Y, Kobayashi H, Galbraith DW (1995) Green-fluorescent protein as a new vital marker in plant cells. Plant J 8:777–784
Shiva Prakash N, Bhojaraja R, Shivbachan SK, Hari Priya GG, Nagraj TK, Prasad V, Srikanth Babu V, Jayaprakash TL, Dasgupta S, Spencer TM, Boddupalli R (2009) Marker-free transgenic corn plant production through cobombardment. Plant Cell Rep 28:1655–1668
Simon-Mateo C, Antonio Garcıa J (2006) MicroRNA-guided processing impairs plum pox virus replication, but the virus readily evolves to escape this silencing mechanism. J Virol 80: 2429–2436
Simpson GC, Filipowcz W (1996) Splicing of pre-cursors to mRNA in higher plants: mechanism, regulation and sub-nuclear organization of the spliceosomal machinery. Plant Mol Biol 32:1–41
Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symp Soc Exp Biol 11:118–131
Smith RH, Hood EE (1995) *Agrobacterium tumefaciens* transformation of monocotyledons. Crop Sci 35:301–309
Somers DA, Rines HW, Gu W, Kaeppler HF, Bushnell WR (1992) Fertile, transgenic oat plants. Bio/Technol 10:1589–1594
Somers DA, Samac DA, Olhoft PM (2003) Recent advances in legume transformation. Plant Physiol 131:892–899
Soyfer VN (1980) Hereditary variability of plants under the action of exogenous DNA. Theor Appl Genet 58:225–235
Sreekala C, Wu L, Gu K, Wang D, Tian D, Yin Z (2005) Excision of a selectable marker in transgenic rice Oryza sativa L using a chemically regulated Cre/loxP system. Plant Cell Rep 24:86–94
Sripriya R, Raghupathy V, Veluthambi K (2008) Generation of selectable marker-free sheath blight resistant transgenic rice plants by efficient co-transformation of a cointegrate vector T-DNA and a binary vector T-DNA in one *Agrobacterium tumefaciens* strain. Plant Cell Rep 27: 1635–1644
Sripriya R, Sangeetha M, Parameswari C, Veluthambi B, Veluthambi K (2011) Improved *Agrobacterium*-mediated cotransformation and selectable marker elimination in transgenic rice by using a high copy number pBin19-derived binary vector. Plant Sci 180:766–774
Stachel SE, Zambryski PC (1989) Generic trans-kingdom sex? Nature 340:190–191

Sunikumar G, Rathore KS (2001) Transgenic cotton factors influencing *Agrobacterium*-mediated transformation and regeneration. Mol Breed 8:37–52

Tabashnik BE, van Rensburg JB, Carriere Y (2009) Field-evolved insect resistance to Bt crops: definition, theory and data. J Econ Entomol 102:2011–2025

Tabashnik BE, Gassmann AJ, Crowder DW, Carriere Y (2008) Field-evolved resistance to Bt toxins Nat. Biotechnol 26:1074–1076

Tamura T, Hara K, Yamaguchi Y, Koizumi N, Sano H (2003) Osmotic stress tolerance of transgenic tobacco expressing a gene encoding a membrane-located receptor-like protein from tobacco plants. Plant Physiol 131:454–462

Tenllado F, Llave C, Dıaz-Ruız JR (2004) RNA interference as a new biotechnological tool for the control of virus diseases in plants. Virus Res 102:85–96

Thirukkumarana G, Ntui VO, Khan RS, Nakamura I, Mii M (2010) Generation of phenotypically normal marker-free transgenic plants of *Kalanchoe blossfeldiana* through hairy root induction. Plant Biotechnol J 27:147–153

Thomashow MF, Nutter R, Montoya AL, Gordon MP, Nester EW (1980) Integration and organization of Ti plasmid sequences in crown gall tumors. Cell 19:729–739

Thu TT, Mai TTX, Deade E, Farsi S, Tadesse Y, Angenum G, Jacobs M (2003) *In vitro* regeneration and transformation of pigeonpea *Cajanus cajan* L Mills P. Mol Breed 11:159–168

Tissier AF, Marillonnet S, Klimyuk V, Patel K, Torres MA, Murphy G, Jones JDG (1999) Multiple independent defective Suppressor-mutator transposon insertions in *Arabidopsis*: A tool for functional genomics. Plant Cell 11:1841–1852

Tomes DT, Weissinger AK, Ross M, Higgins R, Drummond BJ, Schaff S, Malone-Schoneberg J, Staebell M, Flynn P, Anderson J, Howard J (1990) Transgenic tobacco plants and their progeny derived by microprojectile bombardment of tobacco leaves. Plant Mol Biol 14:261–268

Trick HN, Finer JJ (1997) SAAT sonication-assisted *Agrobacterium*-mediated transformation. Transgenic Res 6:329–337

Trieu AT, Burleigh SH, Kardailsky IV, Maldonado-Mendoza IE, Versaw WK, Blaylock LA, Shin H, Chiou T-J, Katagi H, Dewbre GR, Weigel D, Harrison MJ (2000) Transformation of Medicago truncatula via infiltration of seedlings or flowering plants with *Agrobacterium*. Plant J 22:531–541

Tuteja N, Verma S, Sahoo RK, Raveendar S (2012) Recent advances in development of marker-free transgenic plants: Regulation and biosafety concern. J Biosci 371:167–197

Untergasser A, Bijl GJM, Liu W, Bisseling T, Schaart JG, Geurts R (2012) One-Step *Agrobacterium* mediated transformation of eight genes essential for *Rhizobium* symbiotic signalling using the novel binary vector system pHUGE. PLoS ONE 710:e47885. doi:10.1371/journal.pone.0047885

Urushibara S, Tozawa Y, Kawagishi-Kobayashi M, Wakasa K (2001) Efficient transformation of suspension-cultured rice cells mediated by *Agrobacterium tumefaciens*. Breed Sci 5:33–38

Usami S, Okamoto S, Takebe I, Machida Y (1988) Factor inducing *Agrobacterium* tumefaciens vir gene expression is present in monocotyledonous plants. Proc Natl Acad Sci USA 853:748–752

Uzé M, Wünn J, Puonto-Kaerlas J, Potrykus I, Sautter C (1997) Plasmolysis of precultured immature embryos improves *Agrobacterium* mediated gene transfer to rice (*Oryza sativa* L.). Plant Sci 1:3087–3095

Vain P, McMullen MD, Finer JJ (1993) Osmotic treatment enhances particle bombardment mediated transient and stable transformation of maize. Plant Cell Rep 12:84–88

van der Krol AR, Mur LA, Beld M, Mol JNM, Stuitje A (1990) Flavonoid genes in petunia addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell 2:291–299

van der Vossen EAG, Gros J, Sikkema A, Muskens M, Wouters D, Wolters P, Pereira A, Allefs S (2005) The Rpi-blb2 gene from *Solanum bulbocastanum* is an Mi-1 gene homolog conferring broad-spectrum late blight resistance in potato. Plant J 44:208–222

Vasil V, Castillo AM, Fromm ME, Vasil IK (1992) Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Bio/Technology 10:667–674

Vassilakos N (2012) Stability of Transgenic Resistance Against Plant Viruses, Transgenic Plants-Advances and Limitations, PhD. Yelda Ozden Çiftçi (Ed.), ISBN: 978-953-51-0181-9, InTech, DOI: 10.5772/33133. Available from: http://www.intechopen.com/books/transgenic-plants-advances-and-limitations/stability-of-transgenic-resistance-against-plant-viruses

Vaucheret H, Béclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, Mourrain P, Palauqui JC, Vernhettes S (1998) Transgene-induced gene silencing in plants. Plant J 16:651–659

Verweire D, Verleyen K, De Buck S, Claeys M, Angenon G (2007) Marker-free transgenic plants through genetically programmed auto-excision. Plant Physiol 145:1220–1231

Vijayachandra K, Palanichelvam K, Veluthambi K (1995) Rice scutellum induces *Agrobacterium* tumefaciens vir genes and T-strand generation. Plant Mol Biol 29:125–133

Waie B, Rajam MV (2003) Effect of increased polyamine biosynthesis on stress responses in transgenic tobacco by introduction of human S-adenosylmethionine gene. Plant Sci 164: 727–734

Walling LL (2000) The myriad plant responses to herbivores. J Plant Growth Regul 19:195–216

Wally O, Punja ZK (2010) Genetic engineering for increasing fungal and bacterial disease resistance in crop plants. GM Crops 14:199–206

Walz A, Zingen-Sell I, Loeffler M, Sauer M (2008) Expression of an oxalate oxidase gene in tomato and severity of disease caused by *Botrytis cinerea* and *Sclerotinia sclerotiorum*. Plant Pathol 57:453–458

Wan Y, Lemaux PG (1994) Generation of large number of independently transformed fertile barley plants. Plant Physiol 104:37–48

Wang AS, Evans RA, Altendorf PR, Hanten JA, Doyle MC, Rosichan JL (2000) A mannose selection system for production of fertile transgenic maize. Plant Cell Rep 19:654–660

Wang K, Drayton P, Frame B, Dunwell J, Thompson J (1995) Whisker-mediated plant transformation an alternative technology. In Vitro Cell Dev Biol-Plant 31:101–104

Wang MB, Abbott DC, Upadhyaya NM, Jacobsen JV, Waterhouse PM (2001) *Agrobacterium* tumefaciens- mediated transformation of an elite Australian barley cultivar with virus resistance and reporter genes. Aust J Plant Physiol 28:149–156

Wang MB, Upadhyaya NM, Brettell RIS, Waterhouse PM (1997) Intron mediated improvement of a selectable marker gene for plant transformation using *Agrobacterium* tumefaciens. J Genet Breed 51:25–334

Wang Y, Chen B, Hu Y, Li J, Lin Z (2005) Inducible excision of selectable marker gene from transgenic plants by the Cre/lox site-specific recombination system. Transgenic Res 14: 605–614

Weber G, Monajembashi S, Greulich KO, Wolfrum J (1988) Genetic manipulation of plant cells and organelles with a laser microbeam. Plant Cell Tissue Organ Cult 12:219–222

Wilmink A, Dons JJM (1993) Selective agents and marker genes for use in transformation of monocotyledonous plants. Plant Mol Biol Rep 11:165–185

Woo HJ, Cho HS, Lim SH, Shin KS, Lee SM, Lee KJ, Kim DH, Cho YG (2009) Auto-excision of selectable marker genes from transgenic tobacco via a stress inducible FLP/FRT sitespecific recombination system. Transgenic Res 18:455–465

Wright M, Dawson J, Dunder E, Suttie J, Reed J, Kramer C, Chang Y, Novitzky R, Wang H, Artim-Moore L (2001) Efficient biolistic transformation of maize Zea mays L and wheat *Triticum aestivum* L using the phospho-mannose isomerise gene pmi as the selectable marker. Plant Cell Rep 20:429–436

Wu KM (2007) Monitoring and management strategy for *Helicoverpa armigera* resistance to Bt cotton in China. J Invertebr Pathol 95:220–223

Wu X, Shiroto Y, Kishitani S, Ito Y, Toriyama K (2008) Enhanced heat and drought tolerance in transgenic rice seedlings overexpressing OsWRKY11 under the control of HSP101 promoter. Plant Cell Rep 28:21–30

Xiong L, Ishitani M (2006) Stress signal transduction components, pathways, and network integration. In: Rai AK, Takabe T (eds) Abiotic Stress Tolerance in Plants Toward the Improvement of Global Environment and Food. Springer, Dordercht, The Netherlands, pp 3–29

Xu DQ, Huang J, Guo SQ, Yang X, Bao YM, Tang HJ, Zhang HS (2008) Overexpression of a TFIIIA-type zinc finger protein gene ZFP252 enhances drought and salt tolerance in rice (*Oryza sativa* L.). FEBS Lett 582:1037–1043

Xu K, Xu X, Fukao T, Canlas P, Maghirang-Rodriguez R (2006) Sub1A is an ethylene-response-factor–like gene that confers submergence tolerance to rice. Nature 442:705–708

Yamada M, Morishita H, Urano K, Shiozaki N, Yamaguchi-Shinozaki K, Shinozaki K, Yoshiba Y (2005) Effects of free proline accumulation in petunias under drought stress. J Exp Bot 56: 1975–1981

Yamaguchi-Shinozaki K, Urao T, Iwasaki T, Kiyosue T, Shinozaki K (1994) Function and regulation of genes that are induced by dehydration stress in Arabidopsis thaliana. JIRCAS J 1:69–79

Yang G, Rhodes D, Joly RJ (1996) Effects of high temperature on membrane stability and chlorophyll fluorescence in glycine betaine-deficient and glycine betaine containing maize lines. Aust J Plant Physiol 23:437–443

Ye GN, Stone D, Pang SZ, Creely W, Gonzalez K, Hinchee M (1999) Arabidopsis ovule is the target for *Agrobacterium* in planta vacuum infiltration transformation. Plant J 19:249–257

Yoder JI, Goldsbrough AP (1994) Transformation systems for generating marker-free transgenic plants. Biotechnology 12:263–267

Zambryski PC (1992) Chronicles from the *Agrobacterium*-plant cell DNA transfer story. Annu Rev Plant Physiol Plant Mol Biol 43:465–490

Zelasco S, Ressegotti V, Confalonieri M, Carbonera D, Calligari P, Bonadei M, Bisoffi S, Yamada K, Balestrazzi A (2007) Evaluation of MAT-vector system in white poplar Populus alba L and production of ipt marker-free transgenic plants by 'singlestep transformation'. Plant Cell Tissue Organ Cult 91:61–72

Zhang FL, Takahata Y, Watanabe M, Xu JB (2000) *Agrobacterium* mediated transformation of cotyledonary explants of chined cabbage *Brassica campestris* L. ssp. pekinensis. Plant Cell Rep 19:569–575

Zhang HX, Blumwald E (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Nat Biotechnol 19:765–768

Zhang JZ, Creelman RA, Zhu JK (2004) From laboratory to field. Using information from Arabidopsis to engineer salt, cold, and drought tolerance in crops. Plant Physiol 135:615–621

Zhang J, Tan W, Yang XH, Zhang HX (2008) Plastid-expressed choline monooxygenase gene improves salt and drought tolerance through accumulation of glycine betaine in tobacco. Plant Cell Rep 27:1113–1124

Zhang W, Subbarao S, Addae P, Shen A, Armstrong C, Peschke V, Gilbertson L (2003) Cre/lox-mediated marker excision in transgenic maize Zea mays L plants. Theor Appl Genet 107: 1157–1168

Zhang X, Li H, Zhang J, Zhang C, Gong P, Ziaf K, Xiao F, Ye Z (2011) Expression of artificial microRNAs in tomato confers efficient and stable virus resistance in a cell-autonomous manner. Transgenic Res 20:569–581

Zhang Y, Li H, Ouyang B, Lu Y, Ye Z (2006) Chemical-induced autoexcision of selectable markers in elite tomato plants transformed with a gene conferring resistance to lepidopteran insects. Biotechnol Lett 28:1247–1253

Zhao BY, Lin XH, Poland J, Trick H, Leach J, Hulbert S (2005) A maize resistance gene functions against bacterial streak disease in rice. Proc Natl Acad Sci USA 1021:5383–5388

Zhao ZY, Cai T, Tagliani L, Miller M, Wang NH, Rudert M, Schroeder S, Hondred D, Seltzer J, Pierce D (2000) *Agrobacterium* mediated sorghum transformation. Plant Mol Biol 44: 789–798

Zhao ZY, Gu W, Cai T, Tagliani L, Hondred D, Bond D, Schroeder S, Rudert M, Pierce D (2001) High throughput genetic transformation mediated by *Agrobacterium* tumefaciens in maize. Mol Breed 8:323–333

Zheng ZL, Nafisi M, Tam A, Li HM, Crowell DN, Chary SN, Schroeder JI, Shen J, Yang Z (2002) Plasma membrane associated ROP10 small GTPase is a specific negative regulator of abscisic acid responses in Arabidopsis. Plant Cell 14:2787–2797

Zhong H, Sun B, Warkentin D, Zhang S, Wu R, Wu T, Sticklen MB (1996) The competence of maize shoot meristems for integrative transformation and inherited expression of transgenes. Plant Physiol 110:1097–1107

Zhou GY, Weng J, Haung J, Qian S, Liu Q (1983) Introduction of exogenous DNA into cotton embryos. Methods Enzymol 101:433–481

Zuo J, Niu QW, Moller SG, Chua NH (2001) Chemical regulated site-specific DNA excision in transgenic plants. Nat Biotechnol 19:157–161

Chapter 6
Distant Hybridisation and Doubled-Haploidy Breeding

Harinder K. Chaudhary, Vineeta Kaila, Shoukat A. Rather, and Tisu Tayeng

Abstract The combination of genomes from diverse genetic backgrounds through wide hybridisation has become very important during the present days of global climate change. However, in some cases it is not possible to recover hybrids with genomes from both the parental species. The elimination of whole chromosome complement of one of the parents from the wide hybrids, that is, uniparental chromosome elimination, has acted as a boon to the crop breeders for rapid genetic upgradation of the crop varieties. This chapter depicts various chromosome elimination approaches of doubled-haploidy breeding in barley, wheat, oats, triticale and potato. The chapter also presents the possible mechanisms of chromosome elimination including its advantages to the other DH breeding systems in crop plants. It also covers various investigations undertaken throughout the world and the efficiency of various chromosome elimination systems in induction of haploids.

Keywords Wheat • Potato • Wheat X maize • Wide hybridisation • Chromosome elimination • Haploid

6.1 Introduction

Crop improvement involves genetic manipulation of plants in a predetermined way, which often utilises the transfer of genes from one source or genetic background to another. When a plant breeder has determined the direction in which a crop is to be improved, the next crucial step is to find a source of the appropriate gene(s) for making the desired change(s). Once an appropriate source (germplasm) has been

H.K. Chaudhary, Ph.D. (✉) • V. Kaila • S.A. Rather • T. Tayeng
Molecular Cytogenetics & Tissue Culture Lab oratory, Department of Crop Improvement, CSK Himachal Pradesh Agricultural University, Palampur 176062, India
e-mail: ctlhkc@rediffmail.com

A. Pratap and J. Kumar (eds.), *Alien Gene Transfer in Crop Plants, Volume 1: Innovations, Methods and Risk Assessment*, DOI 10.1007/978-1-4614-8585-8_6,
© Springer Science+Business Media New York 2014

found, the next step is to transfer the gene(s) to the parent to be improved. In flowering species, the conventional method of gene transfer is by crossing or sexual hybridisation. This procedure causes genes from the two parents to be assembled into a new genetic matrix. It follows that if parents are not genetically compatible, gene transfer by sexual means cannot occur at all. Artificial sexual hybridisation is the most common conventional method of generating a segregating population for selection in breeding of flowering species. In some breeding programmes, the hybrid (F_1) is the final product. However, in most situations, the F_1 is selfed to generate recombinants (F_2) as a result of recombination of the parental genomes or a segregating population, in which selection is practiced. The tools of modern biotechnology now enable and assist the breeders to transfer genes by circumventing the sexual process, that is, without crossing. More significantly, gene transfer can transcend natural reproductive or genetic barriers. Transfers can occur between unrelated plants and even between different species.

The first choice of parents for use in a breeding programme are cultivars and experimental materials with the traits of interest. Most of the time, plant breeders make elite × elite crosses as they are highly adapted and improved materials. Even though genetic gains from such crosses may not always be dramatic, they are nonetheless significant enough to warrant the practice. After exhausting the variability in the elite germplasm as well as in the cultivated species, the breeder may look elsewhere, based on the gene pool concept of Harlan and de Wet (1971). Hybridisation is a strong evolutionary force which can potentially reshape the genetic composition of populations and create novel genotypes that facilitate adaptation to new environments (Stebbins 1950). Crosses involving materials outside the cultivated species are collectively described as wide crosses. When the wide cross involves another species, it is called an interspecific cross. When it involves a plant from another genus, it is called an intergeneric cross. Intra- and interspecific hybridisation are common means of extending the range of variation beyond that displayed by the parental species.

The primary purpose of wide crosses is to improve a species for economic production by transferring one or a few genes, or segment of chromosomes or whole chromosomes, across interspecific or intergeneric boundaries. The genes may condition a specific disease or pest resistance or may be a product quality trait. Combining genomes from diverse backgrounds may trigger a complementary gene action or even introduce a few genes that could produce previously unobserved phenotypes that may be superior to the parental expression of both qualitative and quantitative traits. Wide crosses often produce sterile hybrids. The genome of such hybrids can be doubled to create a new fertile alloploid species, such as triticale. Cytogenetic studies following a wide cross may be used to understand the phylogenic relationships between the parents of a cross.

Interspecific hybridisation provides information on phylogenetic relationships between any two species giving clues with regard to evolutionary patterns. Often generation of such information is based on cross compatibility, chromosome association and pollen fertility. Such information also helps in developing breeding strategies for introgression of genes from related species into economically useful species. As it creates genetic variation, it has great potential for plant improvement (Goodman et al. 1987; Choudhary et al. 2000; Sain et al. 2002). For certain crops, plant breeders in the

twentieth century have increasingly used interspecific hybridisation for gene transfer from a non-cultivated plant species to a crop variety in a related species. Goodman et al. (1987) presented a list of species in which gene transfers have been successful.

Wild relatives may be sources of useful traits for the improvement of crops. From plant breeding point of view, it is desirable to document the possibility of transferring traits to a crop plant from its wild relatives through conventional sexual hybridisation. Sexual exchanges between species as sources of genetic variability to improve crops have been made possible during the last century by the discovery of efficient ways to circumvent the natural barriers to genetic exchange (Goodman et al. 1987). However, inherent problems of specific introgression such as hybrid instability, infertility, non-Mendelian segregations and low levels of intergenomic crossing-over can constitute important limitations (Stebbins 1950). Moreover, features associated with polyploidy or ploidy dissimilarity between species may result in additional constraints for interspecific gene flow (Rieseberg et al. 2000). After a hybrid plant has been successfully recovered, differences in the number or the compatibility of parental chromosomes may cause sterility. Cytogenetic manipulations have been instrumental in obtaining stable gene transfers. Sterility may result from incomplete or unstable pairing of chromosomes during cell division. For a desired gene from the donor to be incorporated into a chromosome of the crop variety, recombination must take place. If the two species are closely related, natural pairing and recombination may occur (Goodman et al. 1987). High pairing affinity contributes so that once the barriers separating the species are overcome, the gene pools of the two genera are interchangeable (Zwierzykowski et al. 1999). Until recently, the results of interspecific hybridisation could only be studied in a fairly indirect manner. One method was to analyse the phenotype of hybrids, such as the symmetry of morphological characters or the viability of pollen or seed. Alternatively, meiosis in hybrids could be studied by light microscopy and the degree of differentiation between hybridising taxa estimated by analyses of chromosome pairing behaviour and meiotic abnormalities (Rieseberg et al. 2000). Although both of these approaches have been extremely valuable, they can only provide glimpses into the complex interactions of alien genes and genomes following genetic recombination. Cytological analyses are usually performed to evaluate the meiotic process in experimental hybrids. Species with close genetic affinity produce hybrids with regular chromosome pairing, while the hybrids of those more distantly related species have meiotic irregularities and are sterile (Marfil et al. 2006). In diploid interspecific hybrids, the meiotic analysis of chromosome association in the F_1 generation shows the genetic homology between the respective pairs of chromosomes. However, in interspecific tetraploid or hexaploid hybrids, chromosome pairing is affected by the number and similarity among genomes.

Interspecific hybrids have the potential to capture hybrid vigour as well as combine traits that do not occur within a single species (Volker and Orme 1988). Because a breeder always wants to add new type of characteristics to the current cultivars, interspecific hybridisation is indispensable to combine diverse gene pools. Thus interspecific or intergeneric hybrids have the enormous potential to extend not only their qualitative but also quantitative traits such as the type of flower, plant phenotypes and other single dominant traits from parent species with an environmental adaptation. While natural hybrids can exist between species whose flowering times overlap, pre- and post-fertilisation barriers hinder the frequency of these hybrids.

Reproductive barriers to wide or distant hybridisation can be divided into two broad groups—premating and postmating. The premating barriers include failure of zygote formation due to fertilisation barriers like pollen–stigma incompatibility and failure of pollen tube to reach the ovary, whereas the postmating barriers comprise failure of zygote development and uniparental chromosome elimination. The uniparental chromosome elimination acts a bane for the transfer of desirable traits from the wild species into the genetic background of target species, that is, cultivated species. But at the same time, it may act as a boon when whole chromosome complement of the wild species is eliminated resulting in the development of haploid plants of the recipient species. The doubled haploids, produced by doubling the chromosome number of the haploids, have been quite efficiently used by the breeders for achieving absolute homozygosity in just 2 years, thereby saving 5 years of varietal development programmes (Fig. 6.1a, b). Moreover, they have helped us in the quick development of mapping populations. The haploids are also useful in the development of transgenics.

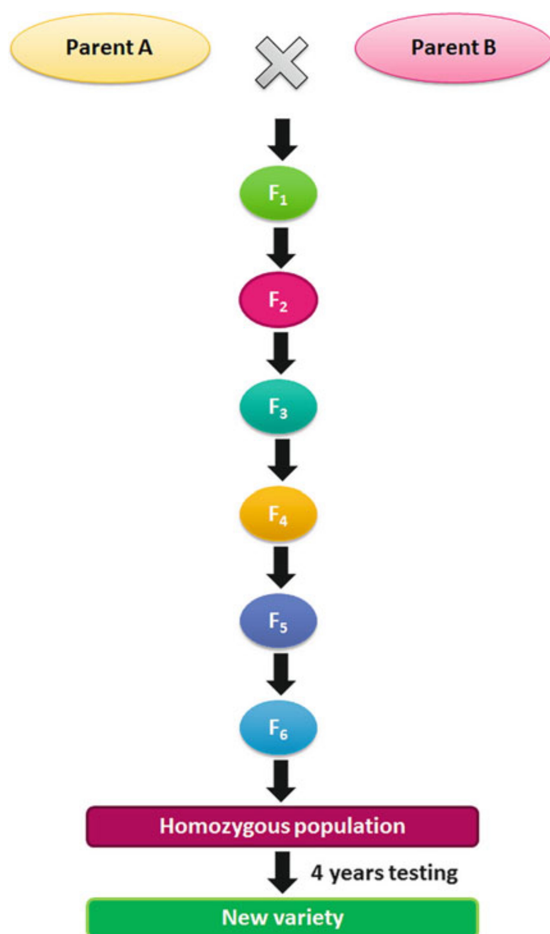

Fig. 6.1 (a and b) Flow chart exhibiting comparison of the conventional and DH breeding approaches

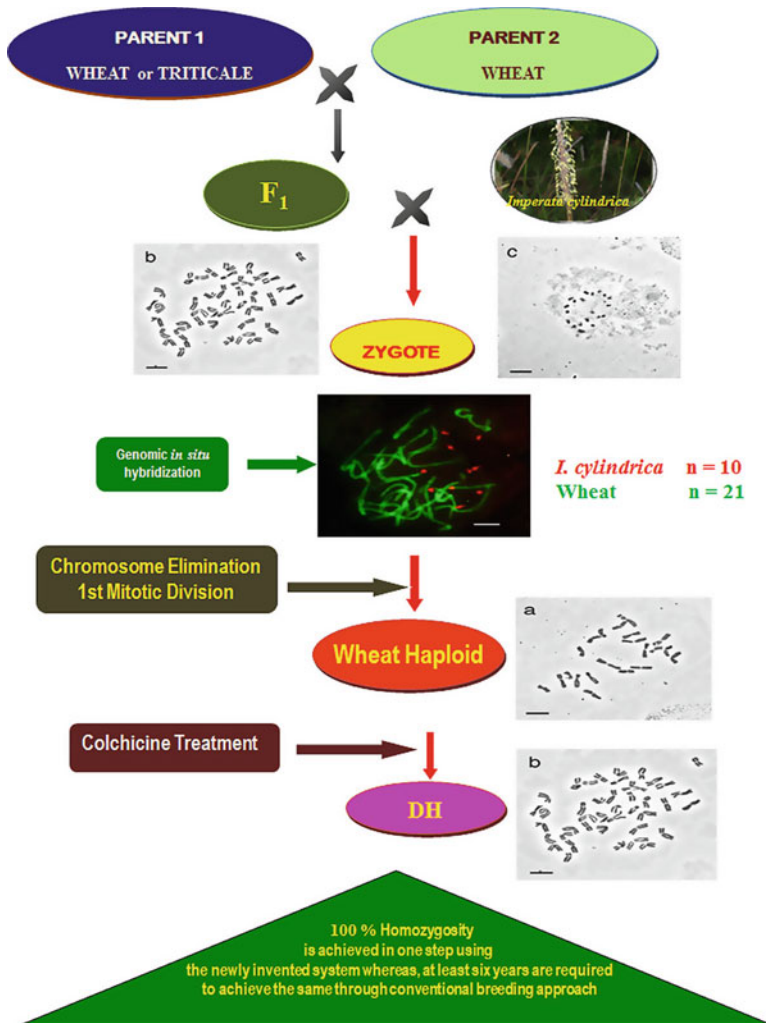

Fig. 6.1 (continued)

The doubled haploidy (DH) breeding following chromosome elimination approach has been exploited in various crops like barley, wheat, oats, triticale, rye and potato, where the other techniques of haploid induction like anther, pollen/microspore culture and ovule culture were not so efficient. In order to apply the DH systems successfully to a breeding programme, any technique should fulfil the following three criteria: (1) DH line(s) should be produced efficiently from all the genotypes, (2) DH should represent a random sample of the parental gametes and (3) DH should be genetically normal and stable (Snape et al. 1986).

Wide crossing between species has been shown to be a very effective and successful method for haploid induction in several species. It exploits haploidy from the

female gametic line and involves both interspecific and intergeneric pollinations. In some interspecific and intergeneric crosses of the Poaceae and Panicoideae, fertilisation is followed by paternal chromosome elimination from the hybrid embryo. In these crosses, the endosperm is either not formed or poorly developed; thus, such embryos do not mature in the caryopsis, and embryo rescue and in vitro culture are necessary. The production of doubled haploids through chromosome elimination occurring during wide crossing is most common in cereals (Laurie et al. 1990).

Several explanations have been proposed to account for uniparental chromosome elimination, viz., difference in timing of essential mitotic processes attributable to asynchronous cell cycling (Gupta 1969) and asynchrony in nucleoprotein synthesis leading to a loss of the most retarded chromosomes (Bennett et al. 1976; Laurie and Bennett 1989). Other hypotheses that have been put forward are the formation of multipolar spindles (Subrahmanyam and Kasha 1973), spatial separation of genomes during interphase (Finch 1983 and Linde-Laursen and von Bothmer 1999) and genome elimination by nuclear extrusions (Gernand et al. 2005, 2006). In addition, degradation of alien chromosomes by host-specific nucleases (Davies 1974), uniparental nondisjunction of anaphase chromosomes (Ishii et al. 2010) and parent-specific inactivation of centromeres (Finch 1983; Jin et al. 2004; Mochida et al. 2004) have been suggested. The actual cellular mechanism involved in the process of uniparental chromosome elimination remains poorly understood.

6.2 Doubled Haploid Through Distant Hybridisation

Various distant hybridisation-mediated doubled haploid techniques are discussed hereunder:

6.2.1 Hordeum vulgare × H. bulbosum

The first method in cereals based on wide crossing following chromosome elimination was *H. vulgare × H. bulbosum*, commonly known as 'bulbosum method' (Stephan 1969; Kasha and Kao 1970; Lange 1971). During early embryogenesis, chromosomes of the wild relative are preferentially eliminated from the cells of developing embryos leading to the formation of the haploid embryos. The endosperm is frequently formed, but its development is usually disturbed; hence, at 12–14 days of pollination, the embryos are excised from developing caryopsis and cultured in vitro. The bulbosum method was the first haploid induction method to produce large number of haploids across most genotypes and this method quickly entered into breeding programmes. Kasha and Kao (1970) presented evidence to show that these haploids are not caused by parthenogenesis but by the elimination of *H. bulbosum* chromosomes. This elimination is under genetic control (Ho and Kasha 1975). Haploids of *H. vulgare* are also obtained when it is used as a male parent in the wide hybridisation programme. This method represents a considerable

advanced approach in the production of barley haploids and it has a number of advantages over anther culture. In particular, haploids can be produced from any cultivar of barley, whereas with anther culture, success is dependent on the genotype.

The parent-specific inactivation of centromeres during the mitosis-dependent process of chromosome elimination in *H. vulgare* × *H. bulbosum* hybrids was confirmed by Sanie et al. (2011). They reported that the loss of centromeric histone H3 (CENH3) from centromeres precedes uniparental chromosome elimination in interspecific barley hybrids. Gernand et al. (2006) studied the mechanism underlying selective elimination of the paternal chromosomes during the development of *H. vulgare* × *H. bulbosum* hybrid embryos that is restricted to an early stage of development. In almost all embryos, most of the *H. bulbosum* chromatin undergoes a fast rate of elimination within 9 days after pollination. According to them, elimination of chromosomes in *H. vulgare* × *H. bulbosum* crosses occurs during mitosis and interphase involves micronucleus formation and progressive heterochromatinisation. The rate of chromosome elimination differs significantly between hybrids, while within each hybrid, differences in mean chromosome number were recorded between and within individual tillers. An increase in temperature from 25 to 30 °C caused a significant increase in the rate of elimination of *H. bulbosum* chromosomes (Humphreys 1978). A high efficiency of *H. bulbosum*-mediated haploid production in barley was achieved using a floret culture technique in which florets pollinated with *H. bulbosum* are cultured on modified N_6 medium containing 0.5 mg/l kinetin and 1.2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) (Chen and Hayes 1989). Toojinda et al. (2000) used bulbosum approach for mapping qualitative and quantitative disease resistance genes in a doubled-haploid population of barley (*H. vulgare*).

Keeping in view the increased efficiency of bulbosum technique of haploid induction in barley as compared to anther culture, the method was extended to wheat where the androgenesis-mediated haploid induction response was very poor and genotype specific.

6.2.2 *Triticum aestivum* × *H. bulbosum*

Haploid wheat plantlets were obtained when 'Chinese Spring' variety of *T. aestivum* (2n = 6x = 42) was crossed with *H. bulbosum* (2n = 2x = 14). This happened as a result of elimination of *H. bulbosum* chromosomes from the interspecifc hybrid during its early embryogenesis (Barclay 1975; Zenketler and Straub 1979). However, this method was not successful with other wheat varieties just like anther culture due to the effect of dominant crossability inhibitor alleles *Kr1*, *Kr2*, *Kr3* and *Kr4* located on 5B, 5A, 5D and 1A chromosome arms (Riley and Chapman 1967; Krolow 1970; Sitch et al. 1985; Zheng et al. 1992) which prevent the entry of *H. bulbosum* pollen tube into the ovary of wheat. The 'Chinese Spring' variety of bread wheat possesses recessive crossability alleles, that is, *kr1 and kr2*. Jalani and Moss (1980) reported that the crossability genes have little effect on pollen germination and on the time taken for the pollen tubes to reach the micropyle. The number of pollen tubes reaching the micropyle is, however, affected by the *Kr*

genes, as high crossable genotypes have more pollen tubes than the low crossable ones. Factors affecting crossability between 'Chinese Spring' wheat and *H. bulbosum* were also found on chromosomes 3A, 3B and 3D (Miller et al. 1983). This system was hence useful to a limited extent due to the sensitivity of the *H. bulbosum* pollen to the crossability inhibitor genes.

6.2.3 Wheat × Maize

Zenkteler and Nitzsche (1984) reported for the first time that embryos were frequently formed when hexaploid wheat was pollinated with maize. Later, their results were confirmed by Laurie and Bennett (1986). They cytologically demonstrated that the maize pollen normally germinated and grew into the wheat embryo sac where the wheat egg was fertilised by the maize pollen. A hybrid zygote with 21 wheat chromosomes and 10 maize chromosomes was produced (Laurie and Bennett 1988). The hybrid zygotes were karyotypically unstable, and the maize chromosomes failed to move towards the spindle poles during cell divisions. Possibly, their centromeres failed to attach to the spindle microtubules due to progressive loss of centromere activity. Resultantly, maize chromosomes were eliminated after three to four mitotic cell divisions forming wheat haploid embryo with n = 21 chromosomes (Laurie and Bennett 1989).

Some earlier studies showed that wheat × maize system has more efficiency of embryo formation as compared to other techniques. For haploid embryo production a system of wheat × maize crossing is widely used due to higher production of haploid embryos as compared to other grass species pollination systems (Inagaki and Tahir 1990; Kisana et al. 1993; Inagaki and Mujeeb-Kazi 1995). This system is fast, economically viable, easy in application and more efficient than others due to low level of genotype specificity (Cherkaoui et al. 2000).

The maize chromosome elimination system in wheat is insensitive to crossability inhibitor genes (Laurie and Bennett 1989) and it enables the production of large number of haploids from any genotype including those recalcitrant to androgenesis (Inagaki et al. 1998; David et al. 1999; Cherkaoui et al. 2000; Chaudhary et al. 2002; Singh et al. 2004; Pratap et al. 2006). Several other investigations of haploid wheat production through wide crossing have since been reported (Laurie and Bennett 1989; Laurie and Reymondie 1991; Matzk and Mahn 1994; Suenaga 1994; Morshedi and Darvey 1995). It appears that a wide range of wheat and maize genotypes can be used to produce haploid wheats, although there is evidence to suggest that the efficiency of production is variable (Suenaga 1994). Haploid production efficiency is affected by the proportion of pollinated florets which develop haploid embryos. Yields of haploid embryos have been reported to be as high as 53 % (Morshedi and Darvey 1995) and as low as 1 % (Suenaga and Nakajima 1989) depending upon a wide range of variables. Factors that affect the yield of haploid embryos include genotypic differences between individual wheat and maize lines (lnagaki and Tahir 1990; Suenaga 1994; Chaudhary et al. 2002; Sharma et al. 2005;

Pratap and Chaudhary 2007; Dhiman et al. 2012), the timing and use of exogenous growth substances to stimulate ovule development (Suenaga and Nakajima 1989) and environmental factors (especially temperature) during and after pollination.

Laurie and Bennett (1989) reported that all maize chromosomes were lost during the first three cell division cycles in most embryos. All embryos with four or more cells had micronuclei, showing that embryo development was dependent on fertilisation. The only primary endosperm metaphase obtained in the experiment had 42 wheat and 10 maize chromosomes, and the presence of micronuclei in most developing endosperms showed that at least 85 % were of hybrid origin.

Zhang et al. (1996) comparatively analysed the embryogenesis in wheat × maize hybrids and self-pollinated wheat plants using paraffin sectioning. They reported that development of embryo is not accompanied by the formation of an endosperm and the endosperm nuclei remain free in the cytoplasm, fail to advance into the cellular stage and degenerate later.

Pratap et al. (2005) evaluated the comparative efficiency of anther culture and maize-mediated system of haploid induction in wheat and triticale genotypes. They reported that haploid plantlet formation was significantly higher through maize-mediated approach as compared to androgenesis in both wheat and triticale genotypes. Auxin analogues play a key role in the induction and maintenance of haploid wheat embryos. Pratap and Chaudhary (2012) investigated the comparative effect of auxins on induction of polyhaploids in triticale × wheat through wheat × maize system.

Wang et al. (1991) studied the frequency of fertilisation and embryo formation in wheat × maize crosses. Hybrid embryos and endosperms obtained from wheat × maize hybridisation were karyotypically unstable and were characterised by rapid elimination of the maize chromosomes to produce haploid wheat embryos. Hence, the reduced genotypic specificity, absence of albinism and ease of application make the wheat × maize hybridisation technique more efficient than the anther culture and the *bulbosum* technique for the production of haploids in common wheat. Accordingly, Inagaki and Tahir (1991), Sun et al. (1992) and Kasha et al. (1995) advocated the use of this technique for breeding purpose by raising a large number of wheat haploids.

Suenaga and Nakajima (1993) evaluated 110 wheat DH lines derived from wheat × maize crosses and found that 15 DH lines were variable for two traits like extreme dwarfism, low seed fertility, alteration of spike type and strips. Analysis of variance within and between DH lines showed the presence of heterogeneity/heterozygosity in the DH lines/plants. Limited studies have been conducted on this line. They inferred that most of the variations detected in the DH lines were due to the effect of colchicine treatment. Similarly Kammholz et al. (1998) also found that expected normal segregation pattern for six glutenin loci across the seven crosses indicated that wheat × maize system is stable across the generations and may meet the third criterion proposed by Snape et al. (1986) for practical wheat breeding programmes. Moreover, Lefebvre and Devaux (1996) also reported normal segregation for 1BL–1RS chromosome through wheat × maize system of cross but which deviates from 1:1 in the haploid progenies produced by anther culture. The wheat × maize system was quite efficiently utilised in the development of the first doubled-haploid wheat variety of India (Him Pratham) (Fig. 6.2) by Dr. Harinder

Fig. 6.2 First doubled-haploid wheat variety of India: DH 114 (Him Pratham) developed through chromosome elimination-mediated approach

Kumar Chaudhary of CSK HP Agricultural University, Palampur, Himachal Pradesh, India (Chaudhary 2013).

Inagaki et al. (1997) crossed hexaploid triticale as well as triticale substitution lines with maize. Hexaploid triticales produced embryos at low frequencies (0.0–5.4 %), whereas higher frequencies were obtained in substitution lines with 2D and 4D chromosomes. This gives an indication that the D-genome chromosomes in triticale genetic background have the effect of increasing the frequency of polyploidy production in triticale × maize crosses. However, maize-mediated system was not able to induce any haploids in wheat × rye derivatives (Kishore et al. 2011).

Durum wheat (2n = 4x = 28) or macaroni wheat is the only tetraploid species of wheat of commercial importance that is widely cultivated today. The ploidy level is not a barrier in the production of haploid embryos through wheat × maize system, and haploids were produced in durum wheat using maize as the pollen source (Ahmad and Chowdhry 2005). Haploid seedlings were recovered from *Triticum turgidum* ssp. *turgidum* cv 'Rampton Rivet' pollinated with maize following in vivo treatment of ovaries with 2,4-D for 2 weeks and subsequent embryo culture. The recovery of haploid seedlings from *T. turgidum* ssp. *durum* cv. 'Wakona' pollinated with maize necessitated the addition of $AgNO_3$ to the 2,4-D treatment (O'Donoughue and Bennett 1994). Almouslem et al. (1998) also reported haploid durum wheat production via hybridisation with maize. Ballesteros et al. (2003) analysed the influence of the relative humidity of the environment, when culturing detached tillers during the production of haploid plants in durum wheat by the maize method and they found that low relative humidity increases haploid induction in durum wheat × maize crosses.

The high haploid induction efficiency and genotype non-specificity of wheat × maize system in comparison to anther culture and bulbosum technique

make the system more practicable. However, the flowering times of maize and wheat can be matched under field conditions in subtropical and tropical climates only, while in other areas experiments are run under glasshouse conditions. Keeping this in view, various efforts have been made throughout the world to search for alternative pollen source for haploid induction in wheat whose flowering must synchronise with wheat under natural conditions. Some of the alternative pollen sources for haploid induction in wheat include pearl millet (Ahmad and Comeau 1990; Inagaki and Mujeeb-Kazi 1995; Ohkawa et al. 1992), *Tripsacum dactyloides* (Riera-Lizarazu and Mujeeb-Kazi 1992) and Job's tears (Mochida and Tsujimoto 2001). More recently, *Imperata cylindrica*, a perennial weedy grass has been reported as the most efficient pollen source for the induction of haploids in wheat, wheat × rye and triticale (Chaudhary et al. 2005; Pratap et al. 2005).

6.2.4 Wheat × Tripsacum dactyloides

To extend the crossing cycle duration, Riera-Lizarazu and Mujeeb-Kazi (1992) performed intergeneric crosses of *T. aestivum*, *T. turgidum* L. and *T. turgidum* × *Aegilops squarrosa* L. (*T. tauschii*) synthetic hexaploids ($2n=6x=42$; AABBDD) with *Tripsacum dactyloides* ($2n=2x=36$) as a pollen donor which resulted in progenies that were polyhaploids of the Triticeae parents, presumably due to elimination of the *Tripsacum dactyloides* chromosomes during early embryo development. Embryo recovery frequencies were 20.6 % for *T. aestivum* cultivars, 26.8 % for *T. turgidum* cultivars and 23.5 % for the synthetic hexaploids. Plant regeneration ranged between 66.7 and 78.5 % over the three maternal crossing groups. As with maize, polyhaploid production in the Triticeae with *Tripsacum* is dependent upon a post-pollination treatment with 2,4-D to promote embryo development and shows no strong genotypic specificity. Limited meiotic analyses for the *T. aestivum* cultivars and synthetic hexaploids gave metaphase I associations characteristic of non-allosyndetic chromosomal pairing. Pollinations with *Tripsacum*, together with maize pollinations, offer an extended crossing cycle and in addition extend the range of alien species for producing polyhaploids in the Triticeae.

6.2.5 Wheat × Pearl Millet

Pearl millet (*Pennisetum glaucum*) is the most widely grown type of millet. It has been grown in Africa and the Indian subcontinent since prehistoric times. Pearl millet is well adapted to growing areas characterised by drought, low soil fertility and high temperature. Haploid wheat plants were obtained when crossed with pearl millet. The wheat plants retained a single pearl millet chromosome at tillering stage, but this chromosome was eliminated from pollen mother cells prior to and also during gamete formation (Ahmad and Comeau 1990). Laurie (1989) undertook wheat × pearl millet crosses to determine whether fertilisation occurred and any

resulting hybrids were karyotypically stable. Crosses between the hexaploid wheat genotype ‘Chinese Spring’ (*kr1*, *kr2*) and the pearl millet genotype ‘Tift 23BE’ yielded fertilisation in 28.6 % of the 220 florets pollinated. Chromosome counts from zygotes at metaphase confirmed the hybrid origin of the embryos. Three had the expected F_1 combination of 21 wheat and 7 pearl millet chromosomes, and a fourth had 21 wheat and 14 pearl millet chromosomes. The expected F_1 chromosome complement was also found in primary endosperm mitosis. The hybrid embryos were karyotypically unstable and probably lost all the pearl millet chromosomes in the first four cell division cycles. Similar results were obtained using two other wheat genotypes. Crosses between the hexaploid wheat genotype ‘Highbury’, which differs from ‘Chinese Spring’ in having alleles for reduced crossability with rye and *H. bulbosum* at the *Kr1* and *Kr2* loci, and ‘Tift 23BE’ registered fertilisation in 32 % of analysed florets. This was not significantly different from the frequency found in ‘Chinese Spring’, indicating that ‘Tift 23BE’ was insensitive to the action of the *Kr* genes. Crosses between the tetraploid wheat genotype ‘Kubanka’ and ‘Tift 23BE’ showed fertilisation in 48 % of florets.

Inagaki and Hash (1998) produced haploids in bread wheat, durum wheat and hexaploid triticale when crossed with pearl millet. The crossability of bread wheat was found to be higher as compared with maize. Inagaki and Mujeeb-Kazi (1995) compared the frequencies of haploid induction in wheat when crossed with maize, pearl millet and sorghum and they observed that maize-mediated haploid induction frequency was higher as compared to the other two which were found to be genotype specific. Deimling et al. (1994) obtained six embryos from which two doubled-haploid lines resulted after pollination of 48,000 emasculated flowers. One embryo was induced by pearl millet and others with maize. Overall, pearl millet could not show its superiority over the maize system in any case of haploid induction and the system was genotype specific.

6.2.6 Wheat × Job’s Tears

Job’s tears (*Coix lacryma-jobi*) is a tall grain-bearing tropical plant of the family Poaceae native to Southeast Asia but elsewhere cultivated in gardens as an annual. It has been naturalised in the southern United States and the New World tropics. In its native environment, it is grown in higher areas where rice and corn do not grow well. Job’s tears are also commonly sold as Chinese pearl barley in Asian supermarkets, although *C. lacryma-jobi* is not closely related to barley (*Hordeum vulgare*). Job’s tears is a perennial plant which forms several stalks and its pollen can be collected throughout the year when the plant is maintained in a controlled environment. Mochida and Tsujimoto (2001) produced wheat (*Triticum aestivum* L.) haploids by crossing with Job’s tears (*Coix lachryma-jobi* L.) as the pollen parent. Pollination was followed by 2,4-D treatment, detached tiller culture and embryo culture, as described for maize pollination. The frequency of embryo formation was similar to that obtained by crossing wheat with maize pollen.

Fig. 6.3 Spike of *Imperata cylindrica*, the efficient pollen source for haploid induction in wheat

6.2.7 Wheat × Imperata cylindrica

Considering the above chromosome elimination-mediated haploid induction systems, no alternative pollen source was reported to overcome the problems of wheat × maize system viz., non-synchronisation of flowering with wheat naturally and poor performance in producing haploids from triticale × wheat and wheat × rye derivatives (Kishore et al. 2011). These constraints made it imperative to search for some other pollen source. Among all the *Gramineae* genera viz., *Zea mays*, *Sorghum bicolor*, *Pennisetum americanum*, *Setaria italica*, *Festuca arundinacea*, *Imperata cylindrica*, *Cynodon dactylon*, *Lolium temulentum* and *Phalaris minor* tested for haploid plant production, *I. cylindrica* produced more embryos and haploids over others (Chaudhary et al. 2005; Pratap et al. 2005). Cogon grass (*I. cylindrica*) (Fig. 6.3) is a wild weedy perennial grass (2n = 2x = 20), does not require repeated sowings and its flowering coincides well with that of wheat and triticale under natural conditions. Furthermore, it is available under natural conditions in almost all parts of the world wherever wheat is cultivated. The *I. cylindrica*-mediated chromosome elimination approach of doubled-haploidy breeding is genotype non-specific for hybridisation with any variety of wheat, triticale or their derivatives.

I. cylindrica has been reported to perform significantly better than maize for all the haploid induction parameters in wheat and triticale and their derivatives (Chaudhary 2008a, b, 2012, 2013). Cytological investigation of the wheat × *I. cylindrica* chromosome elimination system has shown that there is no endosperm formation and the elimination of chromosomes of *I. cylindrica* takes place in the first zygotic division

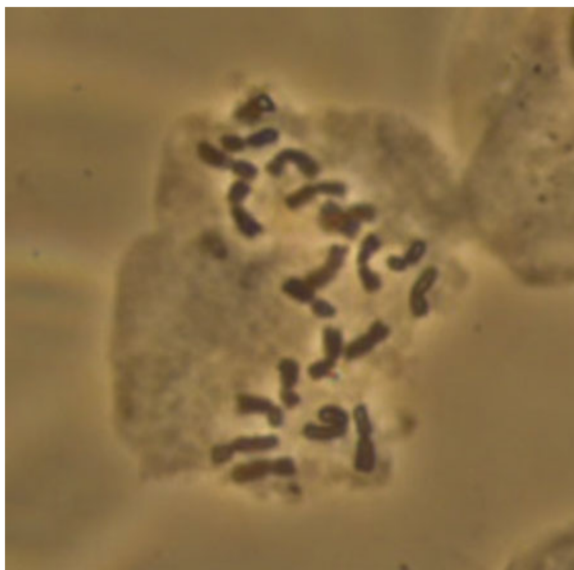

Fig. 6.4 Cytological confirmation of wheat haploid (n = 21) produced from wheat × *Imperata cylindrica* hybridisation (source: Tayeng et al. 2012)

in seed development, thus allowing the production of embryo-carrying seeds (Komeda et al. 2007). Recently, Tayeng et al. (2012) reported that the in vivo application of colchicine (2,000 ppm) enhances the doubled-haploid production efficiency in wheat × *I. cylindrica*-mediated chromosome elimination approach of doubled-haploidy breeding. The haploid chromosome set of wheat (n = 21) obtained after wheat × *I. cylindrica* hybridisation is shown in Fig. 6.4. According to Kaila et al. (2012), the chromosome elimination in wheat × *I. cylindrica* system is being triggered by the B and D genome of wheat. Similar to wheat × maize system, the mean response of wheat and *I. cylindrica* to haploid induction varies from genotype to genotype (Rather et al. 2013). The morphological marker, that is, absence of endosperm in haploid embryo-carrying seeds developed from wheat × *I. cylindrica* hybridisation, can be used quite efficiently to exploit the asynchronous behaviour of anthesis within wheat spikes (Chaudhary et al. 2013) for undertaking this wide hybridisation without emasculation. This endeavour has saved considerable time and energy required otherwise for emasculation in wheat × *I. cylindrica* hybridisation.

6.2.8 *Triticale × Imperata cylindrica*

Triticale is a hybrid of wheat (*Triticum*) and rye (*Secale*) first bred in laboratories during the late nineteenth century. Commercially available triticale is almost a second-generation hybrid, i.e. a cross between two kinds of primary (first cross) triticales. As a rule, triticale combines the yield potential and grain quality of wheat

with the disease and environmental stress tolerance (including stresses related to soil conditions) of rye.

As far as haploid induction following chromosome elimination approach of doubled-haploidy breeding is concerned, Kishore et al. (2011) carried out intergeneric hybridisation using pollen of maize and *I. cylindrica* in wheat–rye-derived backcross (BC_1F_1 and BC_1F_2) generations to study the relative efficiency of the two chromosome elimination systems. The relative efficiency of embryo-carrying seeds ranged from 8 to 30 % with *I. cylindrica*, whereas with maize, no embryo-carrying seeds were obtained. Wedzony et al. (1998) reported the production of haploid embryos in triticale by means of maize-mediated system. However, *I. cylindrica*-mediated system outperformed the maize system in triticale × wheat derivatives in respect of embryo formation and embryo regeneration frequency (Pratap et al. 2005).

6.2.9 *Oat × maize*

The common oat (*Avena sativa*) is a cereal mostly grown for its seed. Oats are suitable for human consumption as oatmeal and rolled oats; however, these are commonly used as livestock feed. Riera-Lizarazu et al. (1996) crossed hexaploid oat (2n = 6x = 42) and maize (2n = 2x = 20) and recovered 90 progenies through embryo rescue. Fifty-two plants (58 %) produced from oat × maize hybridisation were oat haploids (2n = 3x = 21) following maize chromosome elimination. Twenty-eight plants (31 %) were found to be stable partial hybrids with 1–4 maize chromosomes in addition to a haploid set of 21 oat chromosomes (2n = 21 + 1 to 2n = 21 + 4). Ten of the 90 plants produced were found to be apparent chromosomal chimeras, where some tissues in a given plant contained maize chromosomes while other tissues did not, or else different tissues contained a variable number of maize chromosomes. Jing-San and Tie-Gang (1995) crossed naked oat with maize and obtained haploid plants of naked oat.

Factors influencing the rate of caryopsis and haploid embryo production including genotype, post-pollination plant growth regulator application and temperature were investigated (Sidhu et al. 2006). The four growth regulators tested showed significant differences in their capacity to induce caryopsis formation with dicamba producing the highest numbers of caryopses, followed by picloram, 2,4-D and gibberellic acid (GA_3). No significant differences were observed between these growth regulators for their effect on embryo production. The concentration of dicamba was also important and was found to influence caryopsis but not embryo production, with 50 and 100 mg/l dicamba producing significantly more caryopses than 25 or 5 mg/l. Temperature had a significant impact on both caryopsis and embryo production with the magnitude and direction of response depending on genotype. Rates of haploid embryo production observed were between 0.8 and 6.7 % of the pollinated florets. The proportion of haploids, which survived and were successfully doubled with colchicine following transfer to soil, was between 72 and 81 %.

Rines (2002) produced haploids of cultivated oat from wide hybridisation with Panicoideae species, particularly maize. Haploid oat production by the maize wide

cross method appears to be less genotype restricted than haploid production by anther culture. However, the plant recovery frequencies reported to the tune of 1–2 % of maize-pollinated florets are low like those for oat haploid production by anther culture and not yet adequate for routine use in breeding. The oat × maize hybridisation results in novel features in respect of types and reproductive behaviour of plants recovered. These include maize chromosome retention in a portion of the recovered oat plants and partial self-fertility in oat haploid plants. These differences in products can be detrimental in routine production of doubled haploids for breeding, but on the other side the sea normalities have led to the recovery of valuable materials for genetic and genomic studies in both oat and maize. This report details protocols currently in use for recovery and for molecular and cytological characterisation of doubled-haploid oat plants, both with and without added maize chromosomes, from oat × maize hybridisation and describes features of derived plants that make them novel and valuable. Keeping in view the low frequency of haploid recovery through maize-mediated system, there is a need to search some other efficient pollen source so as to enhance the haploid induction efficiency in oat.

6.2.10 Solanum tuberosum × S. phureja

Doubled haploids can be produced from tetraploid genotypes of *S. tuberosum* (cultivated potato) by pollination with the diploid potato species, *S. phureja* (Mendiburu et al. 1974; De Maine 2003). In about 0.5 % of pollinated ovules, both male sperm cells of *S. phureja* take part in the formation of functional endosperm. The best pollinator lines of *S. phureja* were bred for a dominant purple spot embryo marker; thus, seeds containing haploid embryos can be easily distinguished from hybrid *S. tuberosum* × *S. phureja* seeds. Methods of more effective chromosome number duplication were developed more recently, and production of potato can now be obtained by androgenetic methods with a better efficiency (Jacobsen and Ramanna 1994; Rokka et al. 1996; Rokka 2003). Moreover, androgenesis is applicable to a much wider range of *Solanum* species in comparison to crosses with *S. phureja* (Jacobsen and Ramanna 1994; Aziz et al. 1999; Rokka 2003).

Montelongo-Escobedo and Rowe (1969) reported that the superior pollinator in potato haploidy breeding following chromosome elimination approach may be the one that produces a high frequency of restitution sperm nuclei. Dihaploid potatoes can be used for breeding purposes, including alien germplasm introgression or selection at the diploid level, but such plants are not homozygous. Haploids have a significant role in potato breeding programmes, since they enable interspecific hybridisation which would not be otherwise possible due to differences in ploidy levels and endosperm balance numbers. The gene pool of potato can be broadened, and certain valuable traits, such as disease resistance characters from the wild solanaceous species, can be more efficiently introgressed into cultivated potato (Rokka 2009).

6.3 Conclusion

Distant hybridisation has been quite extensively used in various crop improvement programmes as it results in the creation of genetic variation and broadening of the genetic base of the crop plants which helps them to adapt to changing climatic conditions. Among the various barriers involved in the transfer of desirable genes from wild species into the genetic background of modern-day crop varieties, chromosome elimination has been studied to a great extent by the researchers in various crops, especially cereals. The uniparental chromosome elimination in distant or wide hybrids leading to the development of haploids has speeded up the genetic improvement programmes in different crop species as it helps us to achieve the absolute homozygosity in 2 years, thereby saving 5 years of varietal development programmes. Moreover, it assists in the quick development of mapping populations used for molecular studies at various levels. The chromosome elimination-mediated approaches of doubled-haploidy breeding have been used quite efficiently in crops like barley, wheat, oats, triticale and potato, whereas other approaches, viz., androgenesis and gynogenesis, were not so efficient and practicable. The application of any doubled haploid technique to breeding programmes should be able to produce DH lines from all the genotypes, and the DHs should be genetically stable (Snape et al. 1986). The bulbosum technique of haploid induction was found genotype specific just like anther or ovule culture. The wheat × maize system was genotype non-specific, but it failed to produce haploids in wheat × rye derivatives (Kishore et al. 2011). Wheat × *I. cylindrica*, the newly invented system of chromosome elimination, has showed a great promise in producing haploids from wheat, triticale and wheat × rye derivatives (Chaudhary et al. 2005; Chaudhary 2013). Keeping in view that most of the studies in respect of doubled-haploidy breeding following uniparental chromosome elimination have been reported in cereals, the plant breeders should look forward for such types of genotype-non-specific and efficient haploid induction systems in other crops.

Acknowledgment The authors are highly obliged to Prof. Yasuhiko Mukai, Osaka Kyoiku University, Japan, for extending his expertise in obtaining fine resolution of pictures in certain cytogenetic and DH investigations mentioned in this chapter.

References

Ahmad J, Chowdhry MA (2005) Effects of different ploidy levels of wheat (Hexaploids and Tetraploids) on seed set, embryo formation and haploid production in wheat × maize crosses. Pak J Biol Sci 8:1758–1761

Ahmad F, Comeau A (1990) Wheat × pearl millet hybridization: consequence and potential. Euphytica 50:181–190

Almouslem AB, Jouhar PP, Peterson TS, Bommineni VR, Rao MB (1998) Haploid durum wheat production *via* hybridization with maize. Crop Sci 38:1080–1087

Aziz AN, Seabrook JEA, Tai GCC, de Jong H (1999) Screening diploid *Solanum* genotypes responsive to different anther culture conditions and ploidy assessment of anther-derived roots and plants. Am J Potato Res 76:9–16

Ballesteros J, García-Llamas C, Ramirez MC, Martín A, Weber WE (2003) Low relative humidity increases haploid production in durum wheat × maize crosses. Plant Breed 122:276–278

Barclay IR (1975) High frequencies of haploid production in wheat (*Triticum aestivum*) by chromosome elimination. Nature 256:410–411

Bennett MD, Finch RA, Barclay IR (1976) The time rate and mechanism of chromosome elimination in *Hordeum* hybrids. Chromosoma 54:175–200

Chaudhary HK (2008a) Dynamics of wheat × *Imperata cylindrica*—a new chromosome elimination mediated system for efficient haploid induction in wheat. In: Appels R et al (eds) Proceedings of the 11th international wheat genetics symposium, University of Sydney Press, Sydney, Australia, pp 647–650

Chaudhary HK (2008b) Dynamics of doubled haploidy breeding and molecular cytogenetic approaches in bread wheat. In: Taniguchi K, Zhang X (eds) Focus on north-west Himalayan regions. Advances in Chromosome Science, The Society of Chromosome Research, Hiroshima, Japan. vol 3(2). pp 67–69

Chaudhary HK (2012) New frontiers in chromosome engineering for enhanced and high precision crop improvement. In: Proceedings of national seminar on plant cytogenetics: new approaches, Department of Botany, Punjabi University, Patiala, India, pp 35–36

Chaudhary HK (2013) New frontiers in chromosome elimination-mediated doubled haploidy breeding for accelerated and high precision genetic upgradation in wheat. In: Plant and animal genome meeting in the international triticeae mapping initiative workshop, Cornell University, USA

Chaudhary HK, Singh S, Sethi GS (2002) Interactive influence of wheat and maize genotypes on haploid induction in winter × spring wheat hybrids. J Genet Breed 56:259–266

Chaudhary HK, Sethi GS, Singh S, Pratap A, Sharma S (2005) Efficient haploid induction in wheat by using pollen of *Imperata cylindrica*. Plant Breed 124:96–98

Chaudhary HK, Tayeng T, Kaila V, Rather SA (2013) Use of asynchrony in flowering for easy and economical polyhaploid induction in wheat following *Imperata cylindrica*-mediated chromosome elimination approach. Plant Breed. doi:10.1111/pbr.12036

Chen FQ, Hayes PM (1989) A comparison of *Hordeum bulbosum*-mediated haploid production efficiency in barley using in vitro floret and tiller culture. Theor Appl Genet 77:701–704

Cherkaoui S, Lamsaouri O, Chlyah A, Chlyah H (2000) Durum wheat × maize crosses for haploid wheat production: influence of parental genotypes and various experimental factors. Plant Breed 119:31–36

Choudhary BR, Joshi P, Ramarao S (2000) Interspecific hybridization between *Brassica carinata* and *Brassica rapa*. Plant Breed 119:417–420

David JL, Dusautoir JC, Raynauld C, Roumet P (1999) Heritable variation in the ability to produce haploid embryos via pollination with maize and embryo rescue in durum wheat. Genome 42:338–342

Davies DR (1974) Chromosome elimination in inter-specific hybrids. Heredity 32:267–270

De Maine MJ (2003) Potato haploid technologies. In: Maluszynski M et al (eds) Doubled haploid production in crop plants: a manual. Kluwer Academic Publishers, Dordrecht, Netherland, pp 241–247

Deimling S, Flehinghaus-Roux T, Röber F, Schechert A, Roux SR, Geirer HH (1994) Doubled haploid production—now reproducible in rye. In: Abstracts VIIIth international congress of plant tissue and cell culture, p 95

Dhiman R, Rana V, Chaudhary HK (2012) Himalayan maize—potential pollen source for maize mediated system of chromosome elimination approach in DH breeding of bread wheat. Cereal Res Commun 40:246–255

Finch RA (1983) Tissue-specific elimination of alternative whole parental genomes in one barley hybrid. Chromosoma 88:386–393

Gernand D et al (2005) Uniparental chromosome elimination at mitosis and interphase in wheat and pearl millet crosses involves micronucleus formation, progressive heterochromatinization, and DNA fragmentation. Plant Cell 17:2431–2438

Gernand D, Rutten T, Pickering R, Houben A (2006) Elimination of chromosomes in Hordeum vulgare × H. bulbosum crosses at mitosis and interphase involves micronucleus formation and progressive heterochromatinization. Cytogenet Genome Res 114:169–174

Goodman RM, Hauptti H, Crossway A, Knauf VC (1987) Gene transfer in crop improvement. Science 236:48–54

Gupta SB (1969) Duration of mitotic cycle and regulation of DNA replication in *Nicotiana plumbaginifolia* and a hybrid derivative of *N. tabacum* showing chromosome instability. Can J Genet Cytol 11:133–142

Harlan JR, de Wet JMJ (1971) Towards a rational classification of cultivated plants. Taxon 20:509–517

Ho KM, Kasha KJ (1975) Genetic control of chromosome elimination during haploid formation in barley. Genetics 81:263–275

Humphreys MW (1978) Chromosome instability in *Hordeum vulgare* × *H. bulbosum* hybrids. Chromosoma 65:301–307

Inagaki MM, Hash CT (1998) Production of haploids in bread wheat, durum wheat and hexaploid triticale crossed with pearl millet. Plant Breed 117:485–487

Inagaki MN, Mujeeb-Kazi A (1995) Comparison of polyhaploid production frequencies in crosses of hexaploid wheat with maize, pearl millet and sorghum. Breed Sci 45:157–161

Inagaki MN, Tahir M (1991) Efficient production of wheat haploids through intergeneric crosses. TARC Newsletter, Tropical Agriculture Research Centre 2:4

Inagaki MN, Nagamine T, Mujeeb-Kazi A (1997) Use of pollen storage and detached-tiller culture in wheat polyhaploid production through wide crosses. Cereal Res Commun 25:7–13

Inagaki MN, Varughese G, Rajaram S, van Ginkel M, Mujeeb-Kazi A (1998) Comparison of bread wheat lines selected by doubled haploid, single-seed descent and pedigree selection methods. Theor Appl Genet 97:550–556

Inagkai MN, Tahir M (1990) Comparison of haploid production frequencies in wheat varieties crossed with *Hordeum bulbosum* L. and maize. Jpn J Breed 40:209–216

Ishii T, Ueda T, Tanaka H, Tsujimoto H (2010) Chromosome elimination by wide hybridization between Triticeae or oat plant and pearl millet: pearl millet chromosome dynamics in hybrid embryo cells. Chromosome Res 18:821–831

Jacobsen E, Ramanna MS (1994) Production of monohaploids of Solanum tuberosum L. and their use in genetics, molecular biology and breeding. In: Breadshaw JE, Mckay GR (eds) Potato genetics. CAB International, Wallingford, pp 155–170

Jalani BS, Moss JP (1980) The site of action of the crossability genes (*Kr*$_1$, *Kr*$_2$) between *Triticum* and *Secale*. I. Pollen germination, pollen tube growth, and number of pollen tubes. Euphytica 29:571–579

Jing-San S, Tie-Gang L (1995) Naked oat × maize hybridization. Acta Bot Sin 37:255–258

Jin W, Melo JR, Nagaki K, Talbert PB, Henikoff S, Dawe RK, Jiang J (2004) Maize centromeres: organization and functional adaptation in the genetic background of oat. The Plant Cell 16:571–581

Kaila V, Chaudhary HK, Tayeng T, Rather SA (2012) Resolution of genetic mechanism of chromosome elimination in wheat × *Imperata cylindrica* system of doubled haploidy breeding: the genome responsible. In: Proceedings of national seminar on plant cytogenetics: new approaches, Department of Botany, Punjabi University, Patiala, Punjab, India, pp 82

Kammholz SJ, Grams RA, Banks PM, Sutherland MW (1998) Segregation of glutenins in wheat × maize-derived doubled haploid wheat populations. Aust J Agr Res 49:1253–1259

Kasha KJ, Kao KN (1970) High frequency haploid production in barley (*H. vulgare* L.). Nature 225:874–876

Kasha KJ, Yao Q, Sinion E, Hu T, Oro R (1995) Production and application of doubled haploids in crops. In: Proceedings of induced mutations and molecular techniques for crop improvement, Vienna, Austria, pp 23–27

Kisana NS, Nkongolo KK, Quick JS, Johnson DL (1993) Production of doubled haploids by anther culture and wheat × maize method in a wheat breeding programme. Plant Breed 110:96–102

Kishore N, Chaudhary HK, Chahota RK, Kumar V, Sood SP, Jeberson S, Tayeng T (2011) Relative efficiency of the maize and *Imperatacylindrica*-mediated chromosome elimination approaches for induction of haploids of wheat-rye derivatives. Plant Breed 130:192–194

Komeda N, Chaudhary HK, Mukai Y (2007) Cytological evidence for chromosome elimination in wheat × *Imperata cylindrica* hybrids. Genes Genet Syst 82:241–248

Krolow KD (1970) Investigations on compatibility between wheat and rye. Z Pflanzenzuchtung 64:44–72

Lange W (1971) Crosses between *Hordeum vulgare* L. and *H. bulbosum* L. 1.Production, morphology and meiosis of hybrids, haploids and dihaploids. Euphytica 20:14–29

Laurie DA (1989) The frequency of fertilization in wheat × pearl millet crosses. Genome 32:1063–1067

Laurie DA, Bennett MD (1986) Wheat × maize hybridization. Can J Genet Cytol 28:313–316

Laurie DA, Bennett MD (1988) The production of haploid wheat plants from wheat × maize crosses. Theor Appl Genet 76:393–397

Laurie DA, Bennett MD (1989) The timing of chromosome elimination in hexaploid wheat × maize crosses. Genome 32:953–961

Laurie DA, Reymondie S (1991) High frequencies of fertilization and haploid seedling production in crosses between commercial hexaploid wheat varieties and maize. Plant Breed 106:182–189

Laurie DA, Donoughe S, Bennett MD (1990) Wheat × maize and other wide sexual hybrids, their potential for genetic manipulation and crop improvement. In: Gustafson JP (ed) Genetic manipulation in plant development II. Plenum, New York, pp 95–106

Lefebvre D, Devaux P (1996) Doubled haploids of wheat from wheat × maize crosses: genotypic influence, fertility and inheritance of the 1BL-IRS chromosomes. Theor Appl Genet 93: 1267–1273

Linde-Laursen I, von Bothmer R (1999) Orderly arrangement of the chromosomes within barley genomes of chromosome-eliminating *Hordeum lechleri* × barley hybrids. Genome 42:225–236

Marfil CF, Masuelli RW, Davison J, Comai L (2006) Genomic instability in *Solanum tuberosum* × *Solanum kurtzianum* interspecific hybrids. Genome 49:104–113

Matzk F, Mahn A (1994) Improved techniques for haploid production in wheat using chromosome elimination. Plant Breed 113:125–129

Mendiburu AO, Peloquin SJ, Mok DWS (1974) Potato breeding with haploids and 2n gametes. In: Kasha KJ (ed) Haploids in higher plants. Guelph University Press, Guelph, ON, pp 249–259

Miller TE, Reader SM, Gale MD (1983) The effect of homoeologous group 3 chromosomes on chromosome pairing and crossability in *Triticum aestivum*. Can J Genet Cytol 25:634–641

Mochida K, Tsujimoto H (2001) Production of wheat doubled haploids by pollination with Job's Tears (*Coix lachry-majobi* L.). J Hered 92:81–83

Mochida K, Tsujimoto H, Sasakuma T (2004) Confocal analysis of chromosome behaviour in wheat × maize zygotes. Genome 47:199–205

Montelongo-Escobedo H, Rowe PR (1969) Haploid induction in potato: cytological basis for the pollinator effect. Euphytica 18:116–123

Morshedi AR, Darvey NL (1995) High frequency of embryos in wheat × maize crosses. Sabrao J 27:17–22

O'Donoughue LS, Bennett MD (1994) Durum wheat haploid production using maize-wide crossing. Theor Appl Genet 89:559–566

Ohkawa Y, Suenaga K, Ogawa T (1992) Production of haploid wheat plants through pollination of sorghum pollen. Jpn J Breed 42:891–894

Pratap A, Chaudhary HK (2007) Genetic studies on the effect of triticale × wheat F_1s and maize genotypes on haploid induction following wheat × maize system. J Genet Breed 60:119–124

Pratap A, Chaudhary HK (2012) Comparative effect of auxin analogues on induction of polyhaploids in triticale and triticale × wheat hybrids through wheat maize system. Indian J Agric Sci 82:66–70

Pratap A, Sethi GS, Chaudhary HK (2005) Relative efficiency of different *Gramineae* genera for haploid induction in triticale and triticale × wheat hybrids through chromosome elimination technique. Plant Breed 124:147–153

Pratap A, Sethi GS, Chaudhary HK (2006) Relative efficiency of anther culture and chromosome elimination technique for haploid induction in triticale × wheat and triticale × triticale hybrids. Euphytica 150:339–345

Rather SA, Chaudhary HK, Kaila V (2013) Proportional contribution and potential of maternal and paternal genotypes for polyhaploid induction in wheat × *Imperata cylindrica* chromosome elimination approach. Cereal Research Communications. doi:10.1556/CRC.2013.0038

Riera-Lizarazu O, Mujeeb-Kazi A (1992) Polyhaploid production in the Triticeae: wheat × tripsacum crosses. Crop Sci 33:973–976

Riera-Lizarazu O, Rines HW, Phillips RL (1996) Cytological and molecular characterization of oat × maize partial hybrids. Theor Appl Genet 93:123–135

Rieseberg LH, Baird SJE, Gardner KA (2000) Hybridization, introgression and linkage evolution. Plant Mol Biol 42:205–224

Riley R, Chapman V (1967) The inheritance in wheat of crossability with rye. Genet Res 9:259–267

Rines HW (2002) Oat haploids from wide hybridization. In: Maluszynski M et al (eds) Doubled haploid production in crop plants. A manual. Kluwer Academic Publishers, Dordrecht, Netherlands, pp 155–160

Rokka VM (2003) Anther culture through direct embryogenesis in a genetically diverse range of potato (*Solanum*) species and their interspecific and intergeneric hybrids. In: Maluszynski M et al (eds) Doubled haploid production in crop plants. A manual. Kluwer Academic Publishers, Dordrecht, Netherlands, pp 235–245

Rokka VM (2009) Potato haploids and breeding. In: Touraev A et al (eds) Advances in haploid production in higher plants. Springer Science + Business Media B.V, Germany, pp 199–208

Rokka VM, Pietila L, Pehu E (1996) Enhanced production of dihaploid lines *via* anther culture of tetraploid potato (*Solanum tuberosum* L. ssp. *tuberosum*) clones. Am J Potato 73:1–12

Sain RS, Joshi P, Satry EVD (2002) Cytogenetic analysis of interspecific hybrids in genus *Citrullus* (Cucurbitaceae). Euphytica 128:205–210

Sanie M, Pickering R, Kumke K, Nasuda S, Houben A (2011) Loss of centromeric histone H3 (CENH3) from centromeres precedes uniparental chromosome elimination in interspecific barley hybrids. Proc Natl Acad Sci U S A 108:E498–E505

Sharma S, Sethi GS, Chaudhary HK (2005) Influence of winter and spring wheat genetic backgrounds on haploid induction parameters and trait correlations in the wheat × maize system. Euphytica 144:199–205

Sidhu PK, Howes NK, Aung T, Zwer PK, Davies PA (2006) Factors affecting oat haploid production following oat × maize hybridization. Plant Breed 125:243–247

Singh S, Sethi GS, Chaudhary HK (2004) Differential responsiveness of winter and spring wheat genotypes to maize-mediated production of haploids. Cereal Res Commun 32:201–207

Sitch LA, Snape JW, Firman SJ (1985) Intra chromosomal mapping of crossability genes in wheat (*Triticum aestivum*). Theor Appl Genet 70:309–314

Snape JW, Simpson E, Parker BB (1986) Criteria for the selection and use of doubled haploid systems in cereal breeding programs. In: Horn W et al (eds) Genetic manipulation in plant breeding. Walter de Gruiter, New York, pp 217–229

Stebbins GL (1950) Variation and evolution in plants. Columbia University Press, New York

Stephan S (1969) Haploid barley from crosses of *Hordeum bulbosum* (2*x*) × *Hordeum vulgare* (2*x*). Can J Genet Cytol 11:602–608

Subrahmanyam NC, Kasha KJ (1973) Selective chromosomal elimination during haploid formation in barley following interspecific hybridization. Chromosoma 42:111–125

Suenaga K (1994) Doubled haploid system using the intergeneric crosses between wheat (*Triticum aestivum*) and maize (*Zea mays*). Bull Natl Inst Agrobiol Resour 9:83–139

Suenaga K, Nakajima K (1989) Efficient production of haploid wheat (*Triticum aestivum*) through crosses between Japanese wheat and maize (*Zea mays*). Plant Cell Rep 8:263–266

Suenaga K, Nakajima K (1993) Variation on in doubled haploid plants of wheat obtained through wheat (*Triticum aestivum*) × maize (*Zea mays*) crosses. Plant Breed 11:120–124

Sun JS, Lin H, Lu TS, Wang XA, Ren Z, Wing JL, Fang R, Yang C (1992) The production of haploid wheat plants via wheat × maize hybridization. Acta Bot Sin 34:817–821

Tayeng T, Chaudhary HK, Kishore N (2012) Enhancing doubled haploid production efficiency in wheat (*Triticum aestivum* L. em. Thell) by *in vivo* colchicine manipulation in *Imperata cylindrica* mediated chromosome elimination approach. Plant Breed 131:574–578
Toojinda T, Broers LH, Chen XM, Hayes PM, Kleinhofs A, Korte J, Kudrna D, Leung H, Line RF, Powell W (2000) Mapping quantitative and qualitative disease resistance genes in a doubled haploid population of barley (*Hordeum vulgare*). Theor Appl Genet 101:580–589
Volker PW, Orme RK (1988) Provenance trials of *Eucalyptus globulus* and related species in Tasmania. Aust For 51:257–265
Wang JL, Sun JS, Lu TG, Fang R, Cui HR, Cheng SZ, Yang C (1991) Fertilization and embryo development in wheat × maize crosses. Acta Bot Sin 33:674–679
Wedzony M, Marcinska I, Ponitka A, Slusarkiewicz-Jarzina A, Womna J (1998) Production of doubled haploids in Triticale (× *Triticosecale* Wittmack) by means of crosses with maize (*Zea mays*) using Picloram and Dicamba. Plant Breed 117:211–215
Zenketler M, Straub J (1979) Cytoembryological study on the process of fertilization and the development of haploid embryo of *Triticum aestivum* (2n = 42) after crossing with *Hordeum bulbosum* (2n = 14). Z Pflanzenzuchtung 82:36–44
Zenkteler M, Nitzsche W (1984) Wide hybridization experiments in cereals. Theor Appl Genet 68:311–316
Zhang J, Friebe B, Raupp WJ, Harrison SA, Gill BS (1996) Wheat embryogenesis and haploid production in wheat × maize hybrids. Euphytica 90:315–324
Zheng YL, Luo MC, Yen C, Yang JL (1992) Chromosome location of a new crossability gene in common wheat. Wheat Inf Serv 75:36–40
Zwierzykowski Z, Lukaszewski AJ, Naganowska B, Lesniewska A (1999) The pattern of homologous recombination in triploid hybrids of *Lolium multiflorum* with *Festuca pratensis*. Genome 40:720–726

Chapter 7
Role of Molecular Markers

Reyazul Rouf Mir, Javaid Akhter Bhat, Nelofer Jan, Bikram Singh, Ashok Kumar Razdan, Mohd Ashraf Bhat, Ajay Kumar, Ekta Srivastava, and Nupur Malviya

Abstract Over the past two decades tremendous progress has been made in the area of genomics of crop plants, especially evolution of large number of high-throughput cost effective molecular markers and genotyping platforms which have helped to identify, map, and introgress alien genes from the wild backgrounds. The alien genes once mapped have been introgressed into cultivated crop plants through marker-assisted backcrossing (MABC) for improving biotic and abiotic stresses in major crop species including rice, wheat, chickpea, cotton, tomato, etc. Molecular markers associated with favorable alien QTL of wild species have an important role in introgression and tracing of these QTL during their transfer into the background of cultivated species. Thus these have become important for exploitation of alien genes in crop improvement. This chapter discusses the role of molecular markers in crop improvement through alien gene transfer.

R.R. Mir (✉) • J.A. Bhat • B. Singh • A.K. Razdan
Division of Plant Breeding & Genetics, Shere-Kashmir University of Agricultural Sciences & Technology of Jammu (SKUAST-J), Chatha 180009, Jammu, India
e-mail: imrouf2006@gmail.com

N. Jan
Department of Botany, Chaudhary Charan Singh University (CCSU), Meerut 250004, U.P., India

M.A. Bhat
Molecular Biology Laboratory, Division of Plant Breeding & Genetics, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir (SKUAST-K), Shalimar 191121, Kashmir, India

A. Kumar
Department of Plant Sciences, North Dakota State University, Fargo, ND, USA

E. Srivastava • N. Malviya
Division of Crop Improvement, Indian Institute of Pulses Research, Kanpur 208024, India

A. Pratap and J. Kumar (eds.), *Alien Gene Transfer in Crop Plants, Volume 1: Innovations, Methods and Risk Assessment*, DOI 10.1007/978-1-4614-8585-8_7,
© Springer Science+Business Media New York 2014

Keywords AB-QTL analysis • Alien genes • Gene mapping • Introgression • Marker-assisted backcrossing • Molecular markers

7.1 Introduction

Making selections for desired traits such as non-shattering habit, uniform maturity, improved seed fertility, seed dormancy, increased seed number, increase in seed and fruit size, modified plant architecture, and conversion from perennial to annual forms during the process of crop domestication led to a gradual loss in genetic diversity (Tanksley and McCouch 1997). This reduction/loss in genetic diversity during crop domestication could be attributed to: (i) selection by human beings for desirable "domestication related traits", (ii) genetic drift in the form of "domestication bottlenecks" (Eyre-Walker et al. 1998), and (iii) modern plant breeding practices that resulted in the development of high yielding and uniform crop varieties. This reduction in diversity has been more prominent in self-pollinated crops like wheat, where the level of genetic variation in cultivated pool has often been reported to drops below 5 % of that available in nature (Miller and Tanksley 1990; Wang et al. 1992). It makes crops more vulnerable to biotic and abiotic stresses. This may also result in huge losses in yield and quality as observed previously by the attack of shoot fly and Karnal bunt in India (Reif et al. 2005) and the Southern corn leaf blight in the United States (Tanksley and McCouch 1997). Moreover, it reduces chances to identify new and useful gene combinations for crop improvement. To overcome these concerns and for further genetic improvement in crops plants, the natural variation available in wild relatives, landraces, and primitive cultivars of the crop species is required to be harnessed for a rapid and sustainable improvement of crop species for many years (Tanksley and McCouch 1997). Nonetheless, most of our germplasm and wild material stocked in the gene banks could not be exploited efficiently for crop improvement by the scientists/breeders due to: (i) the traditional plant breeding practices, which made improvement of simple traits more feasible rather than the complex traits on the basis of phenotype (Tanksley and McCouch 1997), (ii) F_1 hybrid sterility, (iii) hybrid breakdown, and (iv) linkage drag. Realizing the importance of greater genetic variability in crop improvement, several new tools and technologies have been developed for efficient utilization of these genetic resources. Development in the science of genomics has been one such measure which provided DNA-based molecular markers for use in crop improvement programs. Molecular markers tightly linked to genes/QTL help to reduce the linkage drag associated with alien introgressed segments by transferring only desirable segment/loci from the wild species. These markers also help in identification of favorable QTL/gene alleles controlling agronomically important traits in the background of unadapted germplasm in spite of their inferior phenotype (deVicente and Tanksley 1993; Eshed and Zamir 1995). In this chapter, we have discussed the role of molecular markers for crop improvement through alien introgressions.

7.2 Importance of Alien Gene Introgression

Genetic variation of crop plants is continuously decreasing due to domestication and modern plant breeding practices (Fig. 7.1). This has although resulted in development of high yielding, uniform crop varieties, but it has happened largely at the cost of extinction of primitive ancestors. This problem is especially severe in self-pollinated crops than in cross-pollinated ones (Miller and Tanksley 1990; Wang et al. 1992). Consequently, these uniform and high yielding varieties become more vulnerable to attacks by diseases and insect-pests leading to heavy losses and in some cases, to near extinction of a crop, as is evident from the following two examples in rice and potato.

7.2.1 Grassy Stunt Virus (GSV) Epidemics in Rice

During the early 1970s, before the release of resistant rice cultivars in 1974, GSV epidemics destroyed more than 116,000 ha (287,000 acres) of rice in Indonesia, India, Sri-Lanka, Vietnam, and Philippines. After this, screening of ~17,000

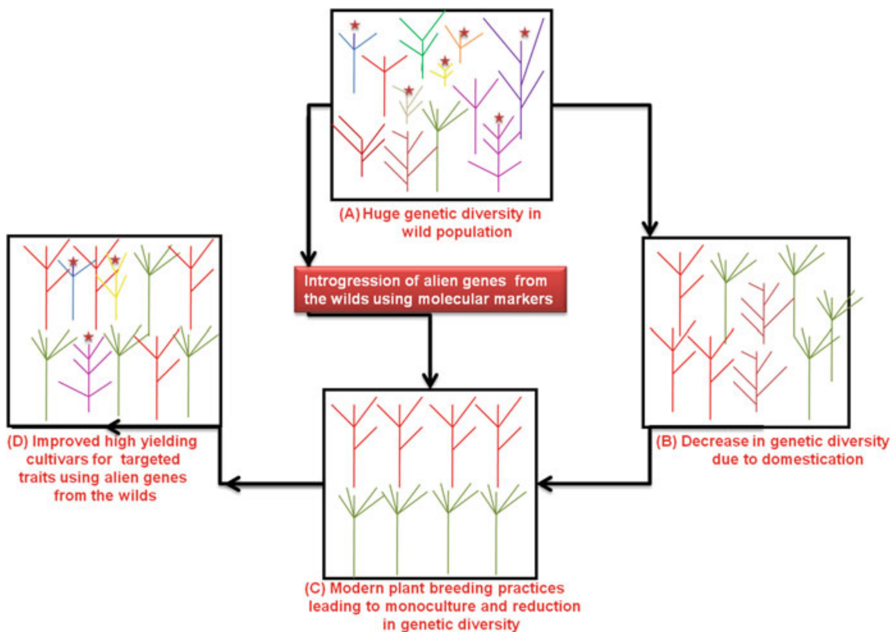

Fig. 7.1 Diagrammatic representation of loss of genetic diversity in crop species (A) due to domestication, (B) modern plant breeding practices, (C) introgression of alien genes from the wild species to improve modern uniform crop varieties (D) for a variety of traits. The Symbol (*asterisk*) indicates the novel genetic variation that has not been selected during domestication and due to modern plants breeding practices, and these novel alleles are now being introgressed from the wild species into elite cultivars with the help of molecular markers to improve them for biotic and abiotic stresses

cultivated and wild rice lines for resistance to GSV for 4 years led to the identification of a population of "*Oryza nivara*", growing wild near Gonda in Uttar Pradesh, India and showing resistant to GSV. This resistance in *O. nivara* for "*grassy-stunt virus strain 1*" was governed by a single resistance gene. This gene was transferred from *O. nivara* into cultivated rice and it is believed that GSV resistant hybrids containing the wild Indian gene are grown across 110,000 km^2 of Asian rice fields (Robert and Prescott-Alen 1983). This is one of the most important examples showing how wild relatives of crop plants came to the rescue of cultivated crops and thus prevented massive crop failure and famine.

7.2.2 Late Blight of Potato

The potato crop was severely attacked by a disease "late blight" caused by the fungus *Phytopthora infestans* in 1945 and 1946. This disease occurred in epidemic throughout northern Europe. In Ireland, where potato was the staple food, a loss of the crop led to wide spread famine. Consequently, human deaths from starvation, combined with emigration to Britain or North America, reduced the population of Ireland from 8.2 million in 1841 to 6.2 million in 1851. However, a breakthrough came in 1908, when the British plant breeder R.N. Salaman found that the wild Mexican species "*Solanum demissum*" and its natural hybrid with the potato *S. edinese* were resistant to late blight. Thus "*S. demissum*" was utilized for the transfer of late blight resistance genes into cultivated pool. It is well documented that out of 586 potato cultivars grown in Europe (West and East, excluding the USSR), 320 have genes from wild species (Stegemann and Loeschcke 1979). Out of 71 cultivars grown in the Soviet Union, 30 contain genes from the wild species (Ross 1979; Robert and Prescott-Alen 1983).

7.3 Evolution of Molecular Maker Technology for Studying Alien Genes

During the last three decades, a variety of DNA-based molecular markers have evolved and helped to study the genetics and molecular breeding of crop plants (Mir and Varshney 2013). Some of these important molecular markers include low-throughput restriction fragment length polymorphisms (RFLPs), medium-throughput random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs) and microsatellite or simple sequence repeats (SSRs), high-throughput single nucleotide polymorphisms (SNPs), diversity array technology (DArT) markers and ultra-high throughput assays for whole-genome genotyping and genotyping-by-sequencing (GBS) (Mir et al. 2013). All these markers have been classified into different categories viz., (i) PCR and non-PCR based,

Table 7.1 Classification of molecular markers on the basis of throughput and generation[a]

Molecular marker	Throughput	Time/generation
Restriction fragment length polymorphisms (RFLPs)	Low-throughput	Past
Random amplified polymorphic DNAs (RAPDs)	Medium-throughput	Past
Amplified fragment length polymorphisms (AFLPs)	Medium-throughput	Past
Simple sequence repeats (SSRs)	Medium-throughput	Present
Single nucleotide polymorphisms (SNPs)	High-throughput	Present
Single feature polymorphisms (SFPs)	High-throughput	Present
Diversity array technology (DArT)	High-throughput	Present
Infinium assays	Ultra high-throughput	Future
Genotyping by sequencing (GBS)	Ultra high-throughput	Future
Reduced-representation libraries (RRLs)	Ultra high-throughput	Future
Restriction site, associated DNA (RAD)-seq	Ultra high-throughput	Future
Complexity reduction of polymorphic sequences (CRoPS)	Ultra high-throughput	Future

[a]Modified from Mir et al. 2013; Mir and Varshney 2013

(ii) hybridization and non-hybridization based, (iii) sequence and non-sequence based, (iv) first, second, third, and fourth generation markers. Additionally, recently these molecular markers have also been classified into (i) past, present, and future molecular markers (Mir and Varshney 2013) and (ii) low-throughput, medium-throughput, high-throughput, and ultra-high throughput molecular markers (Mir et al. 2013; Table 7.1). The last category includes the recently emerged novel array-based, low cost marker systems, including DArTs and single feature polymorphisms (SFPs; Gupta et al. 2008, 2013).

The development of high-throughput array-based markers (e.g., DArTs) overcomes the problems of expensive and laborious scoring of marker panels across target populations in gel-based marker systems (see Gupta et al. 2008, 2013). Among all these markers, SSR markers had become the markers of choice initially because of their various desirable attributes (Gupta and Varshney 2000), but in recent times single nucleotide polymorphisms (SNPs), whose discovery required sequence information have became the markers of choice (in addition to SSRs) due to their abundance and uniform distribution throughout the genomes. All these markers provide most powerful diagnostic tools for the detection of polymorphism either at the specific loci level in the genome or at the whole genome level (Mir and Varshney 2013).

The improvements in screening techniques by molecular markers have been found important in facilitating the tracking of agronomically important genes (Langridge and Chalmers 2004). However, the ultimate approach of studying polymorphism in any crop would be to sequence/resequence the entire genome (or a part of it) of a large number of accessions. This was unimaginable during 1980s and is even still not very cost effective. Therefore, DNA-based molecular markers are employed for detecting and utilization of genetic variation (Collard et al. 2005;

Gupta et al. 2002, 2008). However, the availability of next-generation sequencing (NGS) platforms have revolutionized genomics approaches to biology and have drastically increased the speed at which DNA sequence can be acquired while reducing the costs by several folds. These NGS methods are now using restriction enzyme digestion of target genomes to reduce the complexity for genome-wide genetic marker development and genotyping. The use of restriction enzymes for high-throughput genetic marker discovery and genotyping have several advantages and are becoming the methods of choice for marker discovery (see Davey et al. 2011; Mir and Varshney 2013; Table 7.1). It is anticipated that these emerging technologies will answer many complex biological questions and will help us in identifying recombination breakpoints for linkage mapping or QTL mapping, locating differentially expressed genomic regions between populations for quantitative genetics studies, genotyping large number of progenies for marker-assisted selection (MAS) or resolving the phylo-geography of wild populations. These emerging methods can be grouped into: (1) reduced-representation sequencing, including reduced-representation libraries (RRLs) and complexity reduction of polymorphic sequences (CRoPS), (2) restriction site-associated DNA (RAD)-seq, and (3) low coverage genotyping, including multiplexed shotgun genotyping (MSG) and genotyping by sequencing (GBS). A detailed comparison of various molecular markers and their use has been discussed in detail elsewhere (see Gupta et al. 2008, 2013; Mir et al. 2013; Mir and Varshney 2013).

7.4 Role of Molecular Markers in Alien Gene Introgression

7.4.1 Molecular Markers for Mapping/Tagging of Alien Genes

Several alien genes have been tagged, mapped, and introgressed in major crops like rice and wheat for important traits. A summary of genes/QTLs identified, mapped, and introgressed into elite cultivars in crops like wheat, rice, chickpea, tomato, cotton, etc. have been tabulated in Table 7.2. These useful alien genes have been mapped for crop improvement using several approaches of mapping including single marker analysis (SMA), bulk-segregant analysis (BSA), composite interval mapping (CIM), etc. Markers associated with genes of interest can be deployed to select the genotypes having desirable genes in their background.

7.4.2 Identification of Favorable Alleles/QTL for Complex Traits in the Background of Exotic Germplasm

In general, wild species of crop plants are poor in yield as compared to cultivated species. However, before the advent of molecular markers, it has been shown that use of wild species improves yield of sugar, grains, and fruits.

Table 7.2 A summary of alien QTLs/genes mapped/introgressed from wild species for different traits in some selected crop plants

Crop	Alien gene/QTL	Trait	Source	Reference
Wheat	*LrAC*	Leaf rust resistance	*Caudata*	Riar et al. (2012)
	Qcre.pau-1A, *Qcre.pau-2A*	Cereal cyst nematode resistance	*T. monococcum* L.	Singh et al. (2010)
	Qfe.pau-2A, Qfe.pau-7A	Fe micronutrient	*T. boeoticum*	Tiwari et al. (2009)
	QZn.pau-7A	Zn micronutrient	*T. boeoticum*	Tiwari et al. (2009)
	Lr58	Leaf rust resistance	*A. triuncialis*	Kuraparthy et al. (2011)
	QYrtm.pau-2A, *QYrtb.pau-5A*	Stripe rust resistance	*T. boeoticum*, *T. monococcum*	Chhuneja et al. (2008a)
	LrU1, *LrU2*	Leaf rust resistance	*A. umbellulata*	Chhuneja et al. (2008b)
	YrU1	Stripe rust resistance	*A. umbellulata*	Chhuneja et al. (2008b)
	PmTb7A.1, *PmTb7A.2*	Powdery mildew resistance	*T. boeoticum*	Chhuneja et al. (2012)
Rice	*Bph10.*	BPH resistance	*O. australiensis*	Ishii et al. (1994)
	Bph18(t)	BPH resistance	*O. australiensis*	Jena et al. (2006)
	Qbp1 and Qbp2	BPH resistance	*O. officinalis*	Tan et al. (2004a)
	bph11, *bph12(t)*	BPH resistance	*O. eichingeri*	Hirabayashi et al. (2003)
	Bph20(t), *Bph21(t)*	BPH resistance	*O. minuta*	Rehman et al. (2009)
	Xa21	BB resistance	*O. longistaminata*	Ronald et al. (1992)
	Xa23	BB resistance	*O. rufipogon*	Zhang et al. (1998)
	Xa27	BB resistance	*O. minuta*	Gu et al. (2004)
	Xa29(t)	BB resistance	*O. officinalis*	Tan et al. (2004b)
	Xa30(t)	BB resistance	*O. nivara*	Cheema et al. (2008a)
	Pi-9(t)	Blast resistance	*O. minuta*	Amante-Bordeos et al. (1992)
	Pi-40	Blast resistance	*O. australiensis*	Jeung et al. (2007)
	QTL	Aluminum toxicity tolerance	*O. rufipogon*	Nguyen et al. (2003)

(continued)

Table 7.2 (continued)

Crop	Alien gene/QTL	Trait	Source	Reference
Chickpea	2 QTLs	Ascochyta blight resistance	*C. echinopsermum*	Santra et al. (2000)
	2 QTLs	Ascochyta blight	*C. reticulatum*	Tekeoglu et al. (2002)
	1 QTL	Chickpea rust	*C. reticulatum*	Madrid et al. (2008)
	4 QTLs	Beta-carotene concentration	*C. reticulatum*	Abbo et al. (2005)
	3 QTLs	Seed weight	*C. reticulatum*	Abbo et al. (2005)
Tomato	QTLs	Horticulture traits	*L. pimpinellifolium*	Tanksley et al. (1996)
	QTLs	Fruit weight	*L. peruvianum* 'LA1706'	Fulton et al. (1997)
	2 QTLs	Fruit color	*L. hirsutum* LA407	Kabelka et al. (2004)
	121 QTLs	Agronomic traits	L. hirsutum	Bernacchi et al. (1998)
	QTLs	Ascorbic acid	*S. pennellii*, *S. habrochaites*	Stevens et al. (2007)
	84 QTLs	Yield, processing & fruit quality	*Lycopersicon pennellii* accession LA1657	Frary et al. (2004)
	3 QTL	Bacterial canker resistance	*L. peruvianum* LA2157	Van Heusden et al. (1999)
	1 QTL (*fw2.2*)	Fruit weight	*L. pimpinellifolium*	Alpert et al. (1995)
Cotton	22 QTLs	Fiber elongation	*G. barbadense* cv. 'Pima S6'	Chee et al. (2005a)
	28 QTLs	Fiber length	*G. barbadense* cv. 'Pima S6'	Chee et al. (2005b)
	32 and 9 QTLs	Fiber fineness & micronaire	*G. barbadense* cv. 'Pima S6'	Draye et al. (2005)

BPH= Brown Plant Hopper; BB=Bacterial blight

For example, nobilization of sugarcanes where interspecific crossing of wild species, *Saccharum spontaneum*, was executed with cultivated sugarcane (*S. officinarum*) increased the yield and sucrose content by about 50 %. Similarly, the yield of cultivated oats, *Avena sativa*, increased by 4–7 % by crossing with wild species *A. sterilis* (Frey et al. 1984) and transgressive segregants had >20 % yield increase over the recurrent parent (Lawrence and Frey 1975). The progeny derived from using wild species of tomato, which had small green fruit, showed large red fruits and increased fruit weight (Rick 1974). Improvement in yield was also reported in wheat, oat, barley, sorghum, and maize crops, when wild species were used in crossing (Frey et al. 1984). In maize, crosses with *Tripsacum*, a distant relative of maize, helped to improve yield of popular varieties (Reeves and Bockholt 1964). Yield of chickpea (*Cicer arietinum*) also increased by crossing with wild species *C. echinospermum* (IWC 179) and the derivative lines produced higher yield (19 %), higher biological yield, and heavier seed than the cultivars (Singh and Ocampo 1997). These examples illustrate the possibility of improving yield by up to 20 % using introgressions from wild and related species. However, use of traditional methods only provided limited information on genetic basis of such complex traits, chromosomal locations of QTL and effects of QTL on other traits (epistasis, pleiotropy, or linkage). Availability of dense molecular maps made it possible to locate the genomic regions contributing to a complex trait. Molecular markers help to identify the yield enhancing QTLs among the several QTLs identified in the background of wild species. These yield enhancing-QTL have been mapped by using backcrossed, F_2, introgression lines (ILs) and backcross-double haploid populations in several crops including rice, tomato, wheat, barley, soybean, beans, and capsicum (see Swamy and Sarla 2008 for details). Recently in rice, 40 % alleles of wild species *O. nivara* were identified as trait enhancing alleles in *O. sativa* cv Swarna × *O. nivara* crosses (Swamy et al. 2012). Thus molecular markers associated with these favorable alien genes/QTL of wild species can be traced or introgressed in the background of cultivated species while breeding improved varieties.

7.4.3 Markers for Introgression of Alien Genes through Marker-Assisted Backcrossing (MABC)

The next step followed by mapping of alien genes is their introgression into elite cultivars and improving them for different traits. It is well documented that a large number of alien genes have been introgressed from wild species with the help of molecular markers in all major crop species. These DNA-based molecular markers provided necessary tools for selection of plants in backcross (BC) generations, thus helping to restore the maximum recurrent parent genome along with alien gene from the donor wild parent. This has been done by selecting against the markers from the wild parent outside the region carrying target gene.

Conventional means of transfer of genes from donor into recipient genome takes six backcross generations to yield 99.2 % recurrent parent genome (Allard 1999). However, the most serious concern of breeders is the linkage drag which affects the agronomic performance of the recombinants having most of its genome from the recurrent parent. Molecular markers are used to solve the above two disadvantages associated with the conventional approach through marker-assisted backcrossing (MABC). The MABC involves two steps: (i) foreground selection, which is used to trace the presence of a target gene from the donor parent, and (ii) background selection to track the recovery of the recurrent parent genome. The reconstruction of recurrent genotype through MABC requires only three generations in comparison to more than six generations in traditional backcrossing (Tanksley et al. 1989) and also reduces the chances of linkage drag (Frisch et al. 1999).

Molecular markers, in addition to facilitating alien gene transfer, help to monitor alien gene transfer as well as understand the mechanism of gene transfer. For instance, RFLP markers were used to confirm introgression of 11 out of 12 *O. officinalis* chromosomes in a cross between *O. sativa* and *O. officinalis* in backcross-derived progenies (Jena et al. (1992)). Similarly, introgression of one or two RFLP markers was detected from *O. brachyantha* and *O. granulate* into rice (Brar et al. 1996). MABC breeding has been used successfully for introgression of useful genes from wild and exotic accessions with minimum linkage drag in backcrossing programs. In rice, genes from wild species have been identified in the advanced backcross progenies of crosses involving wild species, *O. australiensis* and *O. brachyantha,* using molecular markers (Ishii et al. 1994; Brar et al. 1996). Substitution lines have been developed with chromosome segments of *O. glaberrima* in *O. sativa* background using RFLP markers during the backcrossing process and constitute useful resources for rice improvement (Doi et al. 2003).

In rice, dozens of alien genes have been characterized, mapped, and transferred into different genetic backgrounds through marker-assisted selection (MAS). Molecular mapping of *Xa-21* gene for bacterial blight using various molecular trait association strategies finally led to the positional cloning of this gene in rice (Song et al. 1995). The gene "*Xa-21*" has been extensively used in molecular breeding programs and more than a dozen rice varieties carrying this gene have been already released through MAS programs worldwide (Brar and Singh 2011). Several other bacterial blight genes were also mapped and used in molecular breeding programs aiming at enhancing bacterial blight resistance of rice. Similarly, gene conferring resistance to *BPH resistance (Bph-10(t))* was also mapped on chromosome 12 by studying co-segregation of BPH with molecular markers and thus closely linked markers for this disease could be identified for molecular breeding programs. Similarly, genes for earliness, blast resistance, tungro tolerance, BPH resistance, and tolerance to aluminum toxicity have also been mapped.

In wheat ~30 species have been investigated and found to contain disease resistance genes. Several genes have been successfully transferred into cultivated wheat using molecular markers leading to release of new varieties with enhanced disease resistance. The amount of alien genome transferred in wheat varies from single gene

to chromosome arms and sometimes even whole chromosome (Jones et al. 1995). Introgression, inheritance and mapping of leaf rust gene of *Ae. Caudata* (CC) in cultivated wheat has been reported using bulk segregant analysis with SSR markers in $F_{2:3}$ mapping populations. This led to the mapping of leaf rust resistance gene (*LrAC*) on short arm of chromosome 5D. The gene *LrAC* was found novel homoeo-allele of an orthologue *Lr57* (Riar et al. 2012). Introgression of group 4 and group 7 chromosomes of *Ae. peregrina* into wheat resulted in 100 % enhancement in grain iron and >200 % increase in grain zinc concentration of BC_2F_2 derivatives. The back-cross progenies were tested with SSR markers and the analysis revealed the introgression of 7S, 7U, group 4 and 4S of *Ae. peregrina* (Neelam et al. 2011). Similarly, a major QTL for both grain Zn and Fe on chromosome 7A in a biparental RIL mapping population derived from a cross between *T. boeoticum* × *T. monococcum* was identified (Tiwari et al. 2009). The substitution of group 2 and 7 of *Ae. kotschyi* in wheat has been reported to be responsible for increasing grain Zn and Fe concentration (Tiwari et al. 2010). Among biotic stresses, cereal cyst nematode resistance was mapped by identification of two QTLs on chromosome arms 1AS and 2AS. The QTL/gene on 1AS may be allelic to *Cre5* (gene from *Ae. ventricosa*) when transferred to cultivated wheat using durum wheat as a bridge species. The use of linked molecular markers confirmed the introgression of CCN resistance in F_8 CCN resistant lines and thus these introgression lines could be used in MAS programs to transfer this gene to elite bread wheat cultivars susceptible to CCN.

In an interesting study, successful transfer, characterization, and mapping of cryptic alien introgression from *Ae. geniculata* with new leaf and stripe rust resistance genes *Lr57* and *Yr40*. In the induced homoeologous chromosome pairing between wheat chromosome 5D and $5M^g$ of *Ae. geniculata* (U^gM^g) and characterization of rust resistant BC_2F_5 and BC_3F_6 progenies identified three introgressions. Molecular mapping revealed that the cryptic alien introgression that confers resistance to leaf and stripe rust comprised <5 % of the chromosome arm 5DS, while genetic mapping using F_2 segregating population showed monogenic and dominant inheritance. Previously mapped RFLP markers on the chromosome arm 5DS showed co-segregation with the rust diseases in F_2 population and mapping locations of these two genes suggested that the leaf and stripe rust resistance genes were new and were designated as *Lr57* and *Yr40* (Kuraparthy et al. 2007a). Similarly, a cryptic introgression from *Ae. triuncialis* into bread wheat were detected using molecular markers. Genetic mapping in a segregating $F_{2:3}$ mapping population showed that rust resistance due to this introgression is controlled by single gene and some selected RFLP markers and one SSR marker could clearly discriminate the resistant lines from the susceptible ones closely linked with this gene. Using bulk segregant analysis, it was proved that the introgressed segment belongs to chromosome arm 2BL, which indicated that the leaf rust resistance gene is new, hence designated as *Lr58* (Kuraparthy et al. 2007b).

Linkage mapping of two adult plant stripe rust resistance genes/QTLs were undertaken in a mapping population derived from a cross between *T. monococcum* × *T. boeoticum*, followed by transfer of one of the genes into bread wheat (Chhuneja

et al. 2008a). The introgressed genes once identified were transferred into different genetic backgrounds for the improvement of concerned diseases. For instance, leaf rust resistance gene *Lr58* discovered by Kuraparthy et al. (2007b) was utilized in MAS programs by developing PCR based codominant markers. The gene was transferred from *Ae. triuncialis* into “Jagger” and “Overley”, two popular winter wheat cultivars of Southern Great Plains, through MABC programs. Screening of BC_3F_4 plants at seedling stage confirmed that the resistance to rust in these progenies was due to the presence of gene “*Lr58*” (Kuraparthy et al. 2011). For powdery mildew disease in wheat, two genes/QTLs flanked by SSR/DArT markers were identified on chromosome 7A and are being deployed into bread wheat cultivars through MABC for development of wheat cultivars with improved powdery mildew resistance (Chhuneja et al. 2012).

7.4.4 Introgressed Alien Gene from the Wild Species and Linkage Drag

Interspecific and even intergeneric crossed have been attempted with good success in majority of crop species to bring/transfer novel genes for different agronomic traits from wild species. However, one needs to take care while attempting to transfer targeted traits from wild species because several undesirable traits also get transferred, the phenomenon known as linkage drag during alien introgression. This linkage drag during the process of alien introgression is due to suppression in recombination at the target gene region and therefore recombination-based approaches cannot be used in the dissection of the target genes (Gill et al. 2011). Precise transfer of genes into cultivated species from wild species without or less linkage drag can be achieved by integrated approaches of cytology, gene expression analysis, conventional and molecular breeding involving use of molecular markers (see Brar and Singh 2011). In case of wheat, it has been shown successfully that the problem can be circumvented and resistance gene can be isolated from an alien introgression using a combination of cytology and gene expression analysis (Cao et al. 2011). Compensating transfer, which involves the induction of homeologous chromosome pairing and thus transfer, takes place between homeologous chromosomes only. This has been demonstrated successfully in the transfer of *LrAC* from WL711 into PBW343 with no apparent linkage drag (Riar et al. 2012). Therefore, one of the major consideration in transferring alien genes is to selectively transfer agronomically important genes from wild species without linkage drag (Brar and Khush 2002; Brar and Singh 2011).

Molecular markers have been found indispensable and used for a variety of purposes including alien gene tagging, mapping, and helping to track and transfer alien genes into different genetic backgrounds. A number of studies have been reported in major crop species including rice and wheat, where alien genes have been tagged and mapped using a variety of molecular markers followed by their

transfer into different genetic backgrounds (Table 7.2). The alien genes reported and transferred in cereals including wheat, rice, and legumes like chickpea have been discussed below.

7.4.4.1 Wheat

In wheat, most of the high yielding varieties possess alien chromosomal introgression from related weedy species. For instance, alien introgression of 1BL·1RS in wheat is due to wheat 1BS and rye 1RS. The rye chromosome arm "1RS" in 1BL·1RS translocation has been found to possess a battery of disease resistance genes including leaf rust (*Lr26*), stem rust (*Sr31*), stripe rust (*Yr9*), and powdery mildew (*Pm8*) as well as genes for adaptation to abiotic stresses, including a robust drought-tolerant root system (Friebe et al 1996; Sharma et al. 2011). A number of other alien genes have been transferred at School of Biotechnology, Punjab Agricultural University, Ludhiana, India from wild relatives for number of diseases including *Lr57*, *Yr40* (for leaf and stripe rust from *Ae. geniculata*), *Lr58* (for leaf rust, powdery mildew, Karnal bunt from *Ae. triuncialis*), *LrU1*, *LrU2*, *YrU1* (for leaf and stripe rust from *Ae. umbellulata*), *LrC* (for leaf and stripe rust from *Ae. caudata*), *LrV*, *YrV* (for leaf and stripe rust from *Ae. variabilis*), some QTLs for leaf and stripe rust, Karnal bunt, powdery mildew, CCN from *T. monococcum* and *T. boeoticum*. For powdery mildew resistance, gene "*Pm21*" has been found effective against a broad spectrum of *Bgt* races in China and other parts of the world. Although large number of alien genes have been deployed in wheat but all of them may not offer durable resistance. For instance, the *Sr31* gene of rye origin, widely deployed in wheat production has recently been broken down by new stem rust races originating in Kenya threatening wheat crop worldwide. Therefore, one has to look for new genes from the wild backgrounds and try to transfer them into cultivated gene pool using different approaches.

7.4.4.2 Rice

Introgression of useful alien genes from wild relatives is routine in rice. The first report of successful alien gene transfer from wild species is introgression of gene for grassy stunt virus resistance from closely related AA genome species "*O. nivara*" to cultivated rice varieties (Khush et al. 1977) and CMS from *O. sativa* ssp. *spontanea* to develop CMS lines for commercial hybrid rice production (Lin and Yuan 1980) Similarly, a dominant gene "*Xa-21*" for bacterial blight was also transferred to rice from *O. longistaminata* (Khush et al. 1990). Crosses were also made between distantly related BB genome and CC genome, which led to the production of several introgression lines with useful genes for resistance to brown plant hopper (BPH), white backed plant hopper (WBPH), and bacterial blight (BB)

(see Brar and Singh 2011). Similarly, genes were introgressed from other wild species with BBCC, CCDD, EE, FF, GG, HHJJ genomes into cultivated rice (for more details see Brar and Khush 1997; Brar and Singh 2011).

7.4.4.3 Chickpea

Most of the food legumes including chickpea suffer from severe crop/yield losses due to damages caused by several biotic and abiotic stresses. Some of the major constraints for chickpea production and productivity include diseases like *Fusarium* wilt and Ascochyta blight, and abiotic stresses, like heat and drought. Several methods have been adopted to breed varieties with enhanced tolerance/resistance to above biotic and abiotic stresses. However, the progress is slow and this could be attributed to unavailability of adequate resistance sources to important stresses within the crop gene pool and the narrow genetic base of chickpea. Therefore, wild crop relatives with broader diversity have been utilized in breeding programs to develop varieties showing enhanced resistance/tolerance to these stresses (Mallikarjuna et al. 2011). For instance, wild *Cicer* species have been used to breed for enhanced resistance against Ascochyta blight, *Fusarium* wilt, Botrytis Gray Mold, *H. armigera* (Pod Borer), Bruchids (*Callosobruchus chinensis*), Cyst nematode (*H. ciceri* Vovlas, Greco, and Divito), protein content and yield, cold tolerance and drought tolerance (Mallikarjuna et al. 2011).

7.4.5 Simultaneous Mapping and Transfer of Alien Genes Through AB-QTL Analysis

The QTL mapping studies have now become common and have been reported in the number of crops like rice, maize, wheat, barley, and other major crops. In all these cases, the QTL mapping involving the identification of QTLs linked to particular marker and their introgression into the elite genotype through MAS are two independent processes. Most of these QTL studies have used early segregating generations (F_2, F_3, and BC_1) for QTL mapping and its detection. The QTLs/genes identified in these early segregating populations loose their effect once they are introgressed into another background of elite genotype, and is because of inter-allelic or epistatic interactions that occur between donor QTL alleles and other donor genes in early mapping generations. However, in the advanced backcross generation due to the recovery of maximum recurrent genome, these interactions get fixed thus leading to possible silencing of the measured QTL effects (Pillen et al. 2003). To solve the above two problems, a new molecular breeding approach has been proposed, which mostly involves two parents: one wild (donor parent) and the other an elite cultivated recurrent parent. This approach simultaneously combines the process of QTL identification in the advanced generation and its introgression into the elite background from the unadapted germplasm. This approach is known as AB-QTL analysis (Tanksley and Nelson 1996, Also see Chap. 1). In this approach the QTL analysis

Table 7.3 AB-QTL analysis studies conducted in crop plants

Crop	Wild/donor parent	Trait studied	Reference
Wheat	Synthetic wheat line (W-7984)	Yield and yield component traits	Huang et al. (2003)
	Synthetic wheat line (XX86)	Agronomic traits	Huang et al. (2004)
	Synthetic wheat line (TA 4152-4)	Yield and yield component traits	Narasimhamoorthy et al. (2006)
	Synthetic hexaploid wheat accessions (Syn022) and (Syn086)	Baking quality traits	Kunert et al. (2007)
	Synthetic wheat accession (Syn022L)	Leaf rust resistance	Naz et al. (2008)
Rice	*O. rufipogon* (IRGC 105491)	Agronomic traits	Xiao et al. (1998)
	O. rufipogon (IRGC 105491)	Yield	Xiao et al. (1996)
	O. rufipogon (IRGC 105491)	Yield, yield component and morphological traits	Thomson et al. (2003)
	O. rufipogon (IRGC 105491)	Agronomic traits	Septiningsih et al. (2003a)
	O. rufipogon (IRGC 105491)	Seed quality traits	Septiningsih et al. (2003b)
	O. rufipogon	Yield, yield component traits	Marri et al. (2005)
	O. grandiglumis	Agronomic traits	Yoon et al. (2006)
	O. rufipogon (IRGC 105491)	Yield and yield component traits	Xie et al. (2008)
	O. rufipogon (IRGC 105491)	Yield and yield component traits	Cheema et al. (2008b)
Barley	*H. vulgare* ssp. spontaneum	Yield and yield component traits	Pillen et al. (2003)
	H. vulgare ssp. spontaneum (ISR42-8)	Powdery mildew, leaf rust and Scald	Von Korff et al. (2005)
	H. vulgare ssp. spontaneum	Agronomic traits	von Korff et al. (2006)
	H. vulgare ssp. spontaneum	Malting quality traits	Von Korff et al. (2008)
Tomato	*L. pimpinellifolium* (LA1589)	Horticulture traits	Tanksley et al. (1996)
	L. peruvianum (LA1706)	Fruit weight	Fulton et al. (1997)
	L. hirsutum (LA1777)	Agronomic traits	Bernacchi et al. (1998)
	L. parviflorum	Horticultural traits	Fulton et al. (2000)
	S. habrochaites	Ascorbic acid	Stevens et al. (2007)
Pepper	*C. frutescens*	Yield related traits	Rao et al. (2003)

is delayed till the latter generations like BC_2 and BC_3, because in these advanced generations most of the recurrent parent genome is recovered and any QTLs/genes identified are free from the epistatic interactions offered by the donor genome. So, the QTLs/genes identified through this approach are believed to perform well in the other genetic backgrounds as well. From almost all these studies it is evident that this approach has the potential in unlocking the favorable alleles from the wild parents. Some of the crops where the AB-QTL analysis has been used for the detection and introgression of QTLs/genes into elite background are tomato, wheat, rice, maize, barley, and cotton (Table 7.3).

7.5 Conclusions

The past two decades have seen tremendous progress in the development of genomic resources across different species of crop plants which have led to a better understanding of the genome structure of crop plants as well as offered new possibilities for their genetic improvement. The availability of next generation sequencing platforms has revolutionized the way genomic resources are developed and also the speed at which a DNA sequence can be acquired at impressively reduced costs. Consequently, an array of DNA-based molecular markers have been developed and employed in not only regular breeding programs but also in successful alien introgressions in several crops including cereals, pulses, oilseeds, ornamentals, and vegetables. Using these markers, numerous alien gene introgressions have been confirmed while through marker-assisted backcrossing new alien introgressions into cultivated background have been materialized. However, keeping in view that the global population is continuously increasing, still more food has to come from plant sources, thereby necessitating exploitation of wild sources for conferring biotic and abiotic stress resistance, nutritional quality and increased yield of crop plants. The AB-QTL approach has to play an increased role in future to breeding cultivars with wider genetic backgrounds while genomic selections aided by genotyping will help in identification of recombination events more precisely. Utilization of specific populations such as introgression and chromosome segment substitution lines, naturally introgressed lines, association mapping populations will further help in mapping of genes/QTLs as well and serve as a useful resource to make selection for desirable recombinants towards development of superior genotypes in different crops. Molecular markers have to still find more use in introgression breeding and this is just a beginning towards sustainable agriculture.

References

Abbo S, Molina C, Jungmann R, Grusa KA, Berkovitch Z, Reifen R, Kahl G, Winter P, Reifen R (2005) Quantitative trait loci governing carotenoid concentration and weight in seeds of chickpea (*Cicer arietinum* L.). Theor Appl Genet 111:185–195

Allard RW (1999) Principles of plant breeding. Wiley, New York

Alpert KA, Grandillo S, Tanksley SD (1995) *fw2.2*: a major QTL controlling fruit weight is common to both red- and green-fruited tomato species. Theor Appl Genet 91:994–1000

Amante-Bordeos A, Sitch LA, Nelson R, Dalmacio RD, Oliva NP, Aswidinnoor H (1992) Transfer of bacterial blight and blast resistance from the tetraploid wild rice *Oryza minuta* to cultivated rice. Theor Appl Genet 84:345–354

Bernacchi D, Beck-Bunn T, Eshed Y, Lopez J, Petiard V, Uhlig J, Zamir D, Tanksley S (1998) Advanced backcross QTL analysis in tomato. I. Identification of QTLs for traits of agronomic importance from *Lycopersicon hirsutum*. Theor Appl Genet 97:381–397

Brar DS, Khush GS (1997) Alien introgression in rice. Plant Mol Biol 35:35–47

Brar DS, Khush GS (2002) Transferring genes from wild species into rice. In: Kang MS (ed) Quantitative genetics, genomics and plant breeding. CABI, Wallingford, pp 197–217

Brar DS, Singh K (2011) Oryza. In: Kole C (ed) Wild crop relatives: genomics and breeding resources, cereals. Springer, Berlin, Heidelberg, pp 321–365

Brar DS, Dalmacio R, Elloran R, Aggarwal R, Angeles R, Khush GS (1996) Gene transfer and molecular characterization of introgression from wild *Oryza* species into rice. In: Khush GS (ed) Rice genetics III. IRRI, Manila, Philippines, pp 477–486

Cao A, Xing L, Wang X, Yang X, Wang W (2011) Serine/threonine kinase gene *Stpk-V*, a key member of powdery mildew resistance gene *Pm21*, confers powdery mildew resistance in wheat. Proc Natl Acad Sci U S A 108:7727–7732

Chee P, Draye X, Jiang CX, Decanini L, Delmonte TA, Bredhauer R, Smith CW, Paterson AH (2005a) Molecular dissection of interspecific variation between *Gossypium hirsutum* and *Gossypium barbadense* (cotton) by a backcross-self approach: I. Fiber elongation. Theor Appl Genet 111:757–763

Chee P, Draye X, Jiang CX, Decanini L, Delmonte TA, Bredhauer R, Smith CW, Paterson AH (2005b) Molecular dissection of phenotypic variation between *Gossypium hirsutum* and *Gossypium barbadense* (cotton) by a backcross-self approach: III. Fiber length. Theor Appl Genet 111:772–781

Cheema KK, Navtej SB, Mangat GS, Das A, Vikal Y, Brar DS, Khush GS, Singh K (2008a) Development of high yielding *IR64* × *Oryza rufipogon* (Griff.) introgression lines and identification of introgressed alien chromosome segments using SSR markers. Euphytica 160:401–409

Cheema KK, Grewal NK, Vikal Y, Das A, Sharma R, Lore JS (2008b) A novel bacterial blight resistance gene from *Oryza nivara* mapped to 38 Kbp region on chromosomes 4L and transferred to *O. Sativa* L. Genet Res 90:397–407

Chhuneja P, Kaur S, Goel RK, Aghaee-Sarbarzeh M, Prashar M, Dhaliwal HS (2008a) Transfer of leaf rust and stripe rust resistance from *Aegilopes umbellulata* Zhuk. to bread wheat (*Triticum aestivum* L.). Genet Resour Crop Evol 55:849–859

Chhuneja P, Kaur S, Garg T, Ghai M, Kaur S, Prashar M, Bains NS, Goel RK, Keller B, Dhaliwal HS, Singh K (2008b) Mapping of adult plant stripe rust resistance genes in diploid A genome wheat species and their transfer to bread wheat. Theor Appl Genet 116:313–324

Chhuneja P, Kumar K, Strinweis D, Hurni S, Keller B, Dhaliwal HS, Singh K (2012) Identification and mapping of two powdery mildew resistance genes in *Triticum boeoticum* L. Theor Appl Genet 124:1051–1058

Collard BCY, Jahufer MZZ, Brouwer JB (2005) An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: the basic concepts. Euphytica 142:169–196

Davey JW, Hohenlohe PA, Etter PD (2011) Genome-wide genetic marker discovery and genotyping using next-generation sequencing. Nat Genet 12:499–510

deVicente MC, Tanksley SD (1993) QTL analysis of transgressive segregation in an interspecific tomato cross. Genetics 134:585–596

Doi K, Sobrizal, Ikeda K, Sanchez PL, Kurakazu T, Nagai Y (2003) Developing and evaluating rice chromosome segment substitution lines. In: Mew TW, Brar DS, Peng S, Dawe D, Hardy H (eds) Rice science: innovation and impact on livelihood. International Rice Research Institute and CASE and CAAS, Beijing, China, pp 289–296

Draye X, Chee P, Jiang CX, Decanini L, Delmonte TA, Bredhauer R, Smith CW, Paterson AH (2005) Molecular dissection of interspecific variation between *Gossypium hirsutum* and *G. barbadense* (cotton) by a backcross-self approach: II. Fiber fineness. Theor Appl Genet 111: 764–771

Eshed Y, Zamir D (1995) An introgression line population of *Lycopersicon pennellii* in the cultivated tomato enables the identification and fine mapping of yield-associated QTL. Genetics 141:1147–1162

Eyre-Walker A, Gaut RL, Hilton H, Feldman DL, Gaut BS (1998) Investigation of the bottleneck leading to the domestication of maize. Proc Natl Acad Sci U S A 95:4441–4446

Frary A, Fulton TM, Zamir D, Tanksley SD (2004) Advanced backcross QTL analysis of a *Lycopersicon esculentum* × *L. Pennellii* cross and identification of possible orthologs in the Solanaceae. Theor Appl Genet 108:485–496

Frey KJ, Cox TS, Rodgers DM, Bramel-Cox P (1984) Increasing cereal yields with genes from wild and weedy species. In Chopra VL et al (eds), Proceedings of the XV international congress of genetics, vol IV (Genetics, new frontiers). pp 51–68

Friebe B, Jiang J, Raupp WJ, McIntosh RA, Gill BS (1996) Characterization of wheat-alien translocations conferring resistance to diseases and pests: current status. Euphytica 91:59–87
Frisch M, Bohn M, Melchinger AE (1999) Comparison of selection strategies for marker-assisted backcrossing of a gene. Crop Sci 39:1295–1301
Fulton TM, Beck-Bunn T, Emmatty D, Eshed Y, Lopez J, Petiard V, Uhlig J, Zamir D, Tanksley SD (1997) QTL analysis of an advanced backcross of *Lycopersicon peruvianum* to the cultivated tomato and comparisons with QTLs found in other wild species. Theor Appl Genet 95:881–894
Fulton TM, Grandillo S, Beck-Bunn T, Fridman E, Frampton A, Lopez J, Petiard V, Uhlig J, Zamir D, Tanksley SD (2000) Advanced backcross QTL analysis of a *Lycopersicon esculentum* × *Lycopersicon parviflorum* cross. Theor Appl Genet 100:1025–1042
Gill BS, Bernd RF, Frank F (2011) Alien introgressions represent a rich source of genes for crop improvement. Proc Natl Acad Sci U S A 108:7657–7658
Gu K, Tian K, Yang F, Wu L, Sreekala C, Wang D, Wang GL, Yin Z (2004) High-resolution genetic mapping of *Xa27*(t), a new bacterial blight resistance gene in rice, *Oryza sativa* L. Theor Appl Genet 108:800–807
Gupta PK, Varshney RK (2000) The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. Euphytica 113:163–185
Gupta PK, Varshney RK, Prasad M (2002) Molecular markers: principles and methodology. In: Jain SM, Ahloowalia BS, Brar DS (eds) Molecular techniques in crop improvement. Kluwer Academic Publishers, Netherlands, pp 9–54
Gupta PK, Rustagi S, Mir RR (2008) Array-based high-throughput DNA markers for crop improvement. Heredity 101:5–18
Gupta PK, Rustgi S, Mir RR (2013) Array-based high-throughput DNA markers and genotyping platforms for cereal genetics and genomics. In: Gupta PK, Varshney RK (eds) Cereal genomics II. Springer, Berlin, Heidelberg, pp 11–55
Hirabayashi H, Kaji R, Okamoto M, Ogawa T, Brar DS, Angeles ER (2003) Mapping QTLs for brown plant hopper (BPH) resistance introgressed from *O. Officinalis* in rice. In: Khush GS, Brar DS, Hardy B (eds) Advances in rice genetics. International Rice Research Institute, Manila, Philippines, pp 268–270
Huang XQ, Cöster H, Ganal MW, Röder MS (2003) Advanced backcross QTL analysis for the identification of quantitative trait loci alleles from wild relatives of wheat (*Triticum aestivum* L.). Theor Appl Genet 106:1379–1389
Huang XQ, Kempf H, Ganal MW, Röder MS (2004) Advanced backcross QTL analysis in progenies derived from a cross between a German elite winter wheat variety and a synthetic wheat (*Triticum aestivum* L.). Theor Appl Genet 109:933–943
Ishii T, Brar DS, Multani DS, Khush GS (1994) Molecular tagging of genes for brown planthopper resistance and earliness introgressed from *Oryza australiensis* into cultivated rice *O. sativa*. Genome 37:217–221
Jena KK, Khush GS, Kochert G (1992) RFLP analysis of rice (*Oryza sativa L*) introgression lines. Theor Appl Genet 84: 608–616
Jena KK, Jeung JU, Lee JH, Choi HC, Brar DS (2006) High-resolution mapping of a new brown plant hopper (BPH) resistance gene, *Bph18*(t), and marker-assisted selection for BPH resistance in rice (*Oryza sativa* L.). Theor Appl Genet 112:288–297
Jeung JU, Kim BR, Cho YC, Han SS, Moon HP, Lee YT, Jena KK (2007) A novel gene, *Pi40(t)* linked to the DNA markers derived from NBS-LRR motifs confers broad spectrum of blast resistance in rice. Theor Appl Genet 115:1163–1177
Jones SS, Murray TD, Allan RE (1995) The development of disease resistance in wheat. Annu Rev Phytopathol 33:429–443
Kabelka E, Yang W, Francis DM (2004) Improved tomato fruit within an inbred backcross line derived from *Lycopersicon esculentum* and *L. hirsutum* involves the interaction of loci. J Am Soc Hortic Sci 129:250–257
Khush GS, Ling KC, Aquino RC, Aquiero VM (1977) Breeding for resistance to grassy stunt in rice. In: Proceedings of 3rd International Congr. SABRAO. Plant Breeding Papers 1[4] Canberra, Australia, pp 3–9

Khush GS, Bacalangco E, Ogawa T (1990) A new gene for resistance to bacterial blight from *O. longistaminata*. Rice Genet Newsl 7:121–122

Kunert A, Naz AA, Dedeck O, Pillen K, Léon J (2007) AB-QTL analysis in winter wheat: I. Synthetic hexaploid wheat (*T. turgidum* ssp. *dicoccoides* × *T. tauschii*) as a source of favourable alleles for milling and baking quality traits. Theor Appl Genet 115:683–695

Kuraparthy V, Chhuneja P, Dhaliwal HS, Kaur S, Bowden RL, Gill BS (2007a) Characterization and mapping of cryptic alien introgression from *Aegilops geniculata* with new leaf rust and stripe rust resistance genes *Lr57* and *Yr40* in wheat. Theor Appl Genet 114:1379–1389

Kuraparthy V, Sood S, Guedira GB, Gill BS (2011) Development of a PCR assay and marker-assisted transfer of leaf rust resistance gene *Lr58* into adapted winter wheats. Euphytica 180:227–234

Kuraparthy V, Soodae S, Chhunejabe P, Dhaliwal HS, Kaura S, Bowdend RL, Gill BS (2007b) A cryptic wheat—*Aegilops triuncialis* translocation with leaf rust resistance gene *Lr58*. Crop Sci 47:1995–2003

Langridge P, Chalmers K (2004) The principle: identification and application of molecular markers. In: Lorz H, Wenzel G (eds) Biotechnology in agriculture and forestry, molecular marker systems in plant breeding and crop improvement. Springer, Berlin, Heidelberg, pp 129–149

Lawrence PK, Frey KJ (1975) Backcross variability for grain yield in oat species crosses (*Avena sativa* L. × *Avena sterilis* L.). Euphytica 24:77–85

Lin SC, Yuan LP (1980) A mass screening method for testing grassy stunt disease of rice. Hybrid rice breeding in China. In: Innovative approaches to rice improvement. International Rice Research Institute, Manila, Philippines, pp 35–51

Madrid E, Rubiales D, Moral A, Moreno MT, Millan T, Gil J, Rubio J (2008) Mechanism and molecular markers associated with rust resistance in a chickpea interspecific cross (*Cicer arietinum* × *Cicer eticulatum*). Eur J Plant Pathol 121:43–53

Mallikarjuna N, Senapathy S, Jadhav DR, Saxena KB, Sharma HC, Upadhyaya HD, Rathore A, Varshney RK (2011) Progress in the utilization of *Cajanus platycarpus* (Benth.) Maesen in pigeonpea improvement. Plant Breed 130:507–514

Marri PR, Sarla N, Reddy LV, Siddiq EA (2005) Identification and mapping of yield and yield related QTLs from an Indian accession of *Oryza rufipogon*. BMC Genet 6:1471–2156

Miller JC, Tanksley SD (1990) RFLP analysis of phylogenetic relationships and genetic variation in the genus *Lycopersicon*. Theor Appl Genet 80:437–448

Mir RR, Varshney RK (2013) Future prospects of molecular markers in plants. In: Henry RJ (ed) Molecular markers in plants. Blackwell Publishing Ltd., Oxford

Mir RR, Hiremath PJ, Riera-Lizarazu O, Varshney RK (2013) Evolving molecular marker technologies in plants: from RFLPs to GBS. In: Lübberstedt T, Varshney RK (eds) Diagnostics in plant breeding. Springer, Berlin, Heidelberg, pp 229–247

Narasimhamoorthy B, Gill BS, Fritz AK, Nelson JC, Brown-Guedira GL (2006) Advanced backcross QTL analysis of a hard winter wheat × synthetic wheat population. Theor Appl Genet 112:787–796

Naz AA, Kunert A, Lind V, Pillen K, Léon J (2008) AB-QTL analysis in winter wheat: II. Genetic analysis of seedling and field resistance against leaf rust in a wheat advanced backcross population. Theor Appl Genet 116:1095–1104

Neelam K, Rawat N, Tiwari V, Kumar S, Chhuneja P, Singh K, Randhawa G, Dhaliwal H (2011) Introgression of group 4 and 7 chromosomes of *Aegilopes peregrina* in wheat enhances grain iron and zinc density. Mol Breed 28:623–634

Nguyen BD, Brar DS, Bui BC, Nguyen TV, Pham LN, Nguyen HT (2003) Identification and mapping of the QTL for aluminum tolerance introgressed from new source, *Oryza rufipogon* Griff. in to indica rice, (*Oryza sativa* L.). Theor Appl Genet 106:583–593

Pillen K, Zacharias A, Léon J (2003) Advanced backcross QTL analysis in barley (*Hordeum vulgare* L.). Theor Appl Genet 107:340–352

Rao GU, Ben Chaim A, Borovsky Y, Paran I (2003) Mapping of yield-related QTLs in pepper in an interspecific cross of *Capsicum annuum and C. frutescens*. Theor Appl Genet 106:1457–1466

Reeves RG, Bockholt AJ (1964) Modification and improvement of maize inbred by crossing it with *Tripsacum*. Crop Sci 4:7–10

Rehman ML, Jiang W, Chu SH, Qiao Y, Ham TH, Woo MO (2009) High resolution mapping of two rice brown plant hopper resistance genes, *Bph20(t)* and *Bph21(t),* originating from *Oryza minuta*. Theor Appl Genet 119:1237–1244

Reif JC, Zhang P, Dreisigacker S, Warburton ML, van Ginkel M, Hoisington D, Bohn M, Melchinger AE (2005) Wheat genetic diversity trends during domestication and breeding. Theor Appl Genet 110:859–864

Riar AK, Kaur S, Dhaliwal HS, Singh K, Chhuneja P (2012) Introgression of a leaf rust resistance gene from *Aegilopes Caudata* to bread wheat. J Genet 91:1–7

Rick CM (1974) High soluble-solids content in large fruited tomato lines derived from a wild green-fruited species. Hilgardia 42:493

Robert, Prescott-Alen C (1983) Genes from the wild. Russel Press, Nottingham, p 95

Ronald PC, Albano B, Tabien R, Abenes L, Wu K, McCouch S (1992) Genetic and physical analysis of rice bacterial blight resistance locus, *Xa21*. Mol Gen Genet 236:113–120

Ross H (1979) Wild species and primitive cultivars as ancestors of potato varieties. In: Zeven AC, van Harten AM (eds) Proceedings of the conference broadening the genetic base of crops, Pudoc, Wageningen, pp 237–245

Santra DK, Tekeoglu M, Ratnaparkhe M, Kaiser WJ, Muehlbauer FJ (2000) Identification and mapping of QTLs conferring resistance to *Ascochyta* blight in chickpea. Crop Sci 40:1606–1612

Septiningsih EM, Pratsetiyono J, Lubis E, Tai TH, Tjubaryat T, Moeljopawiro S, McCouch SR (2003a) Identification of quantitative trait loci for yield and yield components in an advanced backcross population derived from *Oryza sativa* variety IR64 and the wild relative *O. rufipogon*. Theor Appl Genet 107:1419–1432

Septiningsih EM, Trijatmiko KR, Moeljopawiro S, McCouch SR (2003b) Identification of quantitative trait loci for quality in an advanced backcross population derived from *Oryza sativa* variety IR64 and the wild relative *O. rufipogon*. Theor Appl Genet 107:1433–1441

Sharma S, Xu S, Ehdaie B, Hoops A, Close TJ, Lukaszewaki AJ, Waines JG (2011) Dissection of QTL effects for root traits using a chromosome arm-specific mapping population in bread wheat. Theor Appl Genet 122:759–769

Singh KB, Ocampo B (1997) Exploitation of wild *Cicer* species for yield improvement in chickpea. Theor Appl Genet 95:418–423

Singh K, Chhuneja P, Singh I, Sharma SK, Garg T, Garg M, Keller B, Dhaliwal HS (2010) Molecular mapping of cereal cyst nematode resistance in *Triticum monococcum L.* and its transfer to the genetic background of cultivated wheat. Euphytica 176:213–222

Song WY, Wang GL, Chen LL, Kim HS, Pi YL, Holsten T, Gardner J, Wang B, Zhai WX, Zhu LH, Fauquet C, Ronald P (1995) A receptor kinase like protein encoded by the rice disease resistance gene, *Xa-21*. Science 270:1804–1806

Stegemann H, Loeschcke V (1979) Index of European potato varieties: identification by electrophoretic spectra, national registers, appraisal of characteristic, genetic data. Mitteilungen aus der Biologischen Bundesanstalt fur Land-und Forstwirtschaft, Braunschweig, p 233

Stevens R, Buret M, Duffé P, Garchery C, Baldet P, Rothan C, Causse M (2007) Candidate genes and quantitative trait loci affecting fruit ascorbic acid content in three tomato populations. Plant Physiol 143:1943–1953

Swamy BPM, Sarla N (2008) Yield-enhancing quantitative trait loci (QTLs) from wild species. Biotechnol Adv 26:106–120

Swamy BPM, Kaladhar K, Rani NS, Prasad GSV, Viraktamath BC, Reddy GA, Sarla N (2012) QTL analysis for grain quality traits in 2 BC_2F_2 populations derived from crosses between *Oryza sativa* cv swarna and 2 accessions of *O. nivara*. J Hered 103:442–452

Tan GX, Ren X, Weng QM, Shi ZY, Zhu LL, He GC (2004a) Mapping of a new resistance gene to bacterial blight in rice line introgressed from *O. officinalis*. Yi Chuan Xue Bao 31:724–729

Tan G, Weng QM, Ren X, Huang Z, Zhu LL, He GC (2004b) Two whitebacked planthopper resistance genes in rice share the same loci with those for brown planthopper resistance. Heredity 92:212–217

Tanksley SD, McCouch SR (1997) Seed banks and molecular maps: unlocking genetic potential from the wild. Science 277:1063–1066

Tanksley SD, Nelson JC (1996) Advanced backcross QTL analysis: a method for the simultaneous discovery and transfer of valuable QTLs from unadapted germplasm into elite breeding lines. Theor Appl Genet 92:191–203

Tanksley SD, Young ND, Paterson AH, Bonierbale MW (1989) RFLP mapping in plant breeding: new tools for an old science. Nat Biotechnol 7:257–264

Tanksley SD, Grandillo S, Fulton TM, Zamir D, Eshed Y, Petiard V, Lopez J, Beck-Bunn T (1996) Advanced backcross QTL analysis in a cross between an elite processing line of tomato and its wild relative *L. pimpinellifolium*. Theor Appl Genet 92:213–224

Tekeoglu M, Rajesh PN, Muehlbauer FJ (2002) Integration of sequence tagged microsatellite sites to chickpea genetic map. Theor Appl Genet 105:847–854

Thomson MJ, Tai TH, McClung AM, Lai XH, Hinga EM, Lobos KB, Xu Y, Martinez CP, McCouch SR (2003) Mapping quantitative trait loci for yield, yield components and morphological traits in an advanced backcross population between *Oryza rupogon* and the *Oryza sativa* cultivar Jefferson. Theor Appl Genet 107:479–493

Tiwari VK, Rawat N, Chhuneja P, Neelam K, Aggarwal R, Randhawa GS, Dhaliwal HS, Keller B, Singh K (2009) Mapping of quantitative trait loci for grain iron and zinc concentration in diploid a genome wheat. J Hered 100:771–776

Tiwari VK, Rawat N, Neelam K, Kumar S, Randhawa GS, Dhaliwal HS (2010) Substitutions of 2S and 7U chromosomes of *Aegilops kotschyi* in wheat enhance grain iron and zinc concentration. Theor Appl Genet 121:259–269

Van Heusden AW, Koornneef M, Voorrips RE, Bruggenman W, Pet G, Vrielink van Gingel R, Chen X, Lindhout P (1999) Three QTLs from *Lycopersicon peruvianum* confer a high level of reistance to *Clavibacter michiganensis*. Theor Appl Genet 99:1068–1074

Von Korff M, Wang H, Léon J, Pillen K (2005) AB-QTL analysis in spring barley. I. Detection of resistance genes against powdery mildew, leaf rust and scald introgressed from wild barley. Theor Appl Genet 111:583–590

Von Korff M, Wang H, Léon J, Pillen K (2006) AB-QTL analysis in spring barley: II. Detection of favourable exotic alleles for agronomic traits introgressed from wild barley (*H. vulgare* ssp. *spontaneum*). Theor Appl Genet 112:1221–1231

Von Korff M, Wang H, Léon J, Pillen K (2008) AB-QTL analysis in spring barley: III. Identification of exotic alleles for the improvement of malting quality in spring barley (*H. vulgare* ssp. *spontaneum*). Mol Breed 21:81–93

Wang ZY, Second G, Tanksley SD (1992) Polymorphism and phylogenetic relationships among species in the genus *Oryzae* as determined by analysis of nuclear RFLPs. Theor Appl Genet 83:565–581

Xiao J, Li J, Grandillo S, Ahn S, Yuan L, McCouch SR, Tanksley SD (1996) Genes from wild rice improve yield. Nature 384:223–224

Xiao J, Li J, Grandillo S, Ahn S, Yuan L, Tanksley SD, McCouch SR (1998) Identification of trait-improving quantitative trait loci alleles from a wild rice relative, *Oryza rufipogon*. Genetics 150:899–909

Xie X, Jin F, Song MH, Suh JP, Hwang HG, Kim YG, McCouch SR, Ahn SN (2008) Fine mapping of a yield-enhancing QTL cluster associated with transgressive variation in an *Oryza sativa* × *O. rufipogon* cross. Theor Appl Genet 116:613–622

Yoon DB, Kang KH, Kim HJ, Ju HG, Kwon SJ, Suh JP, Jeong OY, Ahn SN (2006) Mapping quantitative trait loci for yield components and morphological traits in an advanced backcross population between *Oryza grandiglumis* and the *O. sativa* japonica cultivar Hwaseongbyeo. Theor Appl Genet 112:1052–1062

Zhang Q, Lin SC, Zhao BY, Wang CL, Wang WC, Zhou YL (1998) Identification and tagging of a new gene for resistance to bacterial blight (*Xanthomonas oryzae* pv. *oryzae*) from *O.rufipogon*. Rice Genet Newsl 15:138–142

Chapter 8
Molecular Cytogenetics for Identification of Alien Chromosomes and Chromosome Segments

Harinder K. Chaudhary, Vineeta Kaila, and Shoukat Ahmad Rather

Abstract The transfer of alien genes into the genetic background of cultivated crop varieties is becoming an important aspect of modern crop improvement programmes. Introgressive hybridization has widened the genetic base of the present day crop cultivars. This chapter depicts the necessity of molecular cytogenetic tools for identification and characterization of the introgressed alien chromosome segments or genes. There are several instances where different molecular cytogenetics tools including in situ hybridization, FISH, GISH and high resolution mapping have been successfully used to detect alien chromosomes or chromosome segments across different crop species and such alien introgressions have been successful in increasing the economic value of the crop. This chapter discusses many such examples and emphasizes upon the increasing role of molecular cytogenetics in alien gene transfers.

Keywords Cytogenetics • In situ hybridization • FISH • GISH • High resolution mapping • Karyotype

8.1 Introduction

Utilization of plant breeding tools for genetic improvement of crop plants has increased crop productivity worldwide, but has simultaneously eroded their genetic variability (Allard 1996; Hoisington et al. 1999). Most of the modern day cultivars have been derived from similar parents and hence have narrow genetic base that makes them fragile to global climate change and disease and insect epidemics (Tanksley and McCouch 1997; Chan 2010). Wild relatives, on the other hand, maintain a wide range of allelic diversity for traits related to their fitness such as disease

H.K. Chaudhary, Ph.D. (✉) • V. Kaila • S.A. Rather
Molecular Cytogenetics and Tissue Culture Lab oratory, Department of Crop Improvement, CSK HP Agricultural University, Palampur 176062, India
e-mail: ctlhkc@rediffmail.com

A. Pratap and J. Kumar (eds.), *Alien Gene Transfer in Crop Plants, Volume 1: Innovations, Methods and Risk Assessment*, DOI 10.1007/978-1-4614-8585-8_8,
© Springer Science+Business Media New York 2014

resistance and enhanced stress tolerance. Broadening the genetic variability will make crop production more sustainable under various biotic and abiotic stresses which are posing major threat in current and upcoming scenario. Introgressive hybridization, the incorporation of genetic materials from one species into another through wide hybridization and repeated backcrossing, plays an important role in the evolution, genetic modification, and enriching the gene pool of plant species (Anamthawat-Jonsson 2001; Brar and Khush 2005; Jellen and Leggett 2005; Singh 2005; Jauhar et al. 2009). The "advanced backcross" approach allows the transfer of genes controlling useful agronomic traits that are not present in the natural background of crop plants, while introgressing the genomic regions and the potential genes controlling specific traits (Tanksley and Nelson 1996). Plant breeders are interested in introgressing genes conferring desirable traits from wild to crop species in breeding programmes (Zhou et al. 2008). While introduction and integration of alien genetic material in crop plants is considered important, its characterization (physical size and precise location) is more vital so as to confirm the introgression of desirable traits only with no linkage drag. However, it is difficult to incorporate alien chromosomes or chromosome segments due to limited chromosome homology and low rates of recombination among chromosomes of wild and recipient crop species (Mujeeb-Kazi 1998; Navabi et al. 2011). The major hindrance in introgressive hybridization lies in recovering and identifying meiotic recombinant chromosomes. Identification of alien introgressions in plants could be done on the basis of expression of that particular alien chromosome or chromosome segment which results in a distinctive morphological expression (Yang and Chen 2009; Anuradha et al. 2006; Multani et al. 2003). In addition, genetic maps can be useful to establish a relative "ranking" among a series of recombinant products (Donini et al. 1995), but they represent poor indicators of physical distances along chromosomes. Other techniques include utilization of biochemical and molecular markers which successfully identify if the introgressions are incorporated in the genetic background of recipient parent (Zhou et al. 2008; Ceoloni et al. 2005; Niu et al. 2011; Simmonds 1993). Most of the times combination of two or more techniques are used to identify desirable introgressions (Multani et al. 2003); however, with such screening techniques significant information about precise location and behaviour of alien segments introgressed is not available. As mentioned above, introgressive hybridization can also introduce resultant linkage drag that can lead to the simultaneous introduction of undesirable chromosome segments from alien parental species (Brown et al. 2003; Desloire et al. 2003). Such introgressions may remain undetected by utilizing aforesaid methods. Hence, to physically map the alien introgressions cytogenetic techniques have been proposed as complementary tools.

8.2 Conventional Cytogenetics

Cytogenetics is the branch of biology dealing with the study of structure and properties of chromosomes in relation to genetics and their behaviour in somatic cell division during growth and development (mitosis) and germ cell division during

reproduction (meiosis), as well as their influence on phenotype. Apart from structural and developmental aspects, cytogenetics also includes the study of factors that bring about chromosomal changes in a somatic compliment. The conventional cytogenetics includes study of metaphase cells based on staining with basic dyes *viz.,* aceto-carmine, feulgen reagent, alcoholic-hydrochloric acid-carmine (Snow 1963), lacto-propionic-oresin (Dyer 1963), carboral fusin (Darlington and La Cour 1969), etc. that stain the chromosomes and make them distinct from rest of the cell constituents. The chromosomes are studied on the basis of chromosome number, morphology such as chromosome size, arm ratio, and presence of secondary constrictions or the chromosomal behaviour during meiosis (Zhou et al. 2008; Tan et al. 2009a, b). The identification of chromosomes and development of karyotype was easy and highly exploited utilizing conventional cytogentic techniques, especially in species where complete and distinct alien chromosomes are introgressed. However, the conventional staining techniques were not useful when the chromosomes of alien and recipient species were not distinctive, hence, different banding techniques were used to distinguish chromosomes belonging to different species. Chromosome banding techniques were applied on prophase chromosomes which have just started to coil, having distinct euchromatin and heterochromatin region. The heterochromatic region constitutes of the highly repetitive areas which coil faster than the unique euchromatin regions of DNA. The more coiled repetitive sequences are stained darker in comparison to the euchromatin region and thus, on the basis of location of heterochromatin and euchromatin region, the chromosomes could be easily differentiated (Gill and Kimber 1974; Merker 1979; Nakata et al. 1977; Sethi and Plaha 1988). The various chromosome banding techniques include Q-bands, G-bands, C-bands, R-bands, N-bands, H-bands, and T-bands (Casperson et al. 1971; Bostock and Sumner 1978; Kannan and Zilfalil 2009). These classical approaches have proven valuable for chromosome characterization, but were unable to distinguish cryptic alien translocations in the host genome. The development of in situ hybridization (ISH), a molecular cytogenetic technique combining cytology with molecular biology allowed direct visualization of specific alien DNA sequences translocated on host chromosomes (Gill and Friebe 1998; Harper and Cande 2000; Rieseberg et al. 2000; Anamthawat-Jonsson 2001).

8.3 Molecular Cytogenetics

Molecular cytogenetics involves the analysis of genomic compositions and alterations using ISH-based technology. The development of ISH techniques opened up opportunities for cytogenetic analysis of essentially any species, regardless of its inherent chromosome morphology. In plants, the use of radioactive tracer or modified nucleotides (attached to biotin, digoxigenin, or fluorescent moieties) to make ISH probes permits microscopic visualization and localization of complementary sequences in cells, nuclei, and individual chromosomes on metaphase spreads (Buongiorno-Nardelli and Amaldi 1969; John et al. 1969; Gall and Pardue 1969). Molecular cytogenetics is commonly used to map alien introgressions, unique or

low-copy-number sequences, repetitive sequences to produce chromosome recognition cocktails or even whole genome to explore genome relations in polyploid or closely related plant species. The broad applications of molecular cytogenetics in structural, comparative, and functional genomics place plant cytogenetics in a unique position to complement, accelerate, or guide plant-genome research.

Molecular cytogenetics and the methods of ISH, especially with fluorescently labelled probes, have revolutionized our understanding of the structure, function, organization, and evolution of genes and the genome. These methods made it feasible to link the molecular data about DNA sequence with chromosomal and expression information at the tissue, cellular, and sub cellular level and hence, changed the way we apply cytogenetics to agriculture (Schwarzacher and Heslop-Harrison 2000). The above-mentioned characteristics of this technique make molecular cytogenetics an efficient tool to assess the physical amount of exchanged material, which is a critical parameter in evaluating the potential impact of an alien transfer on the recipient genotype. Molecular cytogenetic techniques of nonradioactive ISH, particularly fluorescence in situ hybridization (FISH), represent a very efficient tool for precise estimation of the size of chromosomal segments incorporated and, in some cases, directly select promising lines on this basis for practical utilization (Yamamoto and Mukai 1989).

8.4 Molecular Cytogenetic Techniques

8.4.1 In Situ Hybridization

ISH technique, originally developed by Buongiorno-Nardelli and Amaldi (1969), Gall and Pardue (1969), and John et al. (1969), allows genes or DNA sequences to be directly localized on chromosomes in cytological preparations. In this technique, radioactive-labelled probes were hybridized with immobilized cells either in interphase or metaphase spreads. Upon hybridization, the signals emitted by radioactive-labelled probes were detected by autoradiography. The technique was initially illustrated by the hybridization of ribosomal RNA to the amplified ribosomal genes in oocytes of the toad *Xenopus* (Gall and Pardue 1969). The ISH of RNA to the DNA in a cytological preparation should exhibit a high degree of spatial localization as each RNA species hybridizes only with sequences to which it is complementary. The procedure of ISH requires removal of basic proteins from chromosomes or nuclei as they may interfere with the hybridization process. This method was a better technique for rapid localization of specific DNA sequences on cytological preparations itself; however, it was accompanied by a number of limiting factors such as use of radioisotopes whose shelf life is limited. ISH technique has been used to identify chromosomes in several plant species and special cytogenetic stocks, particularly in wheat and its allied genera. Rayburn and Gill (1985) reported a biotin-labelling technique for the mapping of DNA sequences in plant

chromosomes by means of ISH. The biotin-labelling technique also permits the location of single-copy DNA sequences in plant chromosomes, suggesting that the technique may be as sensitive as the isotope-labelling technique for the detection of hybridization sites. This technique was similarly utilized effectively to identify alien DNA segments in the chromosome spreads of plant species. Lapitan et al. (1986) used ISH of wheat–rye hybrids using biotin-labelled rye repetitive sequences and detected translocations of rye chromatins in 14 wheat chromosomes and suggested it as an efficient and sensitive method of detecting translocations. Simultaneously Ambros et al. (1986) utilized this technique to identify the physical location of T-DNA from *Agrobacterium ryzogenes* into the chromosomes of *Crepis capillaris*. Although, the development of this technique allowed rapid and precise physical localization of the target but only one target could be visualized at one time as autoradiography cannot differentiate between two spots. Also, the spatial resolution of image obtained following ISH was less and obviously safety always remains a prime concern.

8.4.2 Fluorescence In Situ Hybridization

Fluorescent ISH emerged to replace the isotopic ISH as powerful physical DNA mapping technique for detection of specific nucleic acid sequences, localization of unique or low copy number sequences and highly repetitive DNA sequences in the specific regions of the chromosomes by using DNA probes labelled with a marker molecule (fluorophore) that emits fluorescent signals which are visualized using a fluorescent microscope. The technique was initially used in plants by Yamamoto and Mukai (1989). They used biotin-labelled wheat ribosomal RNA gene and 120 bp repeated DNA family of rye to detect monosomic and substitution lines and hybrid between wheat and rye *via* fluorescent detection and suggested that the technique was useful for identification of specific chromosomes of a species or related species and alien chromosomes in their hybrids. Several such probes are cloned and can be used for identification of chromosome in those genomes and their chromosomes (Leitch et al. 1991; Mukai et al. 1993b; Mukai 1995a). In particular, FISH has been successfully applied in diverse breeding strategies with various plant species (Jiang and Gill 1994). The main advantage of FISH is that it allows detection of the extent of introgression across the entire genome in a single hybridization experiment, utilizing the in situ labelling of homologous chromosomes or chromosome regions on the basis of divergent and dispersed repetitive or unique sequences.

The hessian fly resistance imparted to bread wheat *via* two terminal translocations T6BS-6BL-6RL and T4BS-4BL-6RL were detected using fluorescently labelled rye-specific probes pSc119 and pSc74. The study revealed not only the introgressed alien chromatin but the exact break points were also identified. Alien introgressions imparting resistance or tolerance against biotic and abiotic stresses were incorporated into genetic background of wheat from *Thinopyrum bessarabicum* by William and Mujeeb-Kazi (1995) and later from *Th. elongatum* × *Secale cereale* hybrids or amphiploids, *Ae. Variablis*, *Th. bessaracicum*, *Triticum currifolium*,

T. scirpeum, and T. elongatum and these introgressions were diagnosed *via* FISH (Mujeeb-Kazi et al. 1996). Ahmad et al. (2000) reported various types of aberrations including wheat–rye, wheat–wheat, rye–rye, wheat–rye–wheat, rye–wheat–rye translocations by treating wheat–rye hybrids with ionizing radiations, and FISH was used to detect alien-introgression due to induced chromosomal translocations. Similarly, FISH was used to characterize the powdery mildew resistant lines derived from wheat–rye substitution lines and wheat-*Lymus mollis* amphidiploids containing rye chromosomes by Forsstrom et al. (2002). They also successfully detected chromosome rearrangements like pericentric inversion of 1R chromosome in wheat-triticale cross using FISH. Recently, Chen et al. (2012) detected a translocation from *Th. ponticum* using pAs1 probe in a novel semi-dwarf line developed from wheat × *Th. ponticum* and reported that this cryptic translocation could not be detected *via* GISH. In addition to hexaploid wheat, FISH was also used successfully in tetraploid wheats, and Armstrong et al. (1992) used this technique to study segregation of homeologous chromosomes in an amphidiploid of *T. durum* × *Th. distichum*. In rice (*Oryza sativa*), Jiang et al. (1995) mapped *Xa21* gene conferring resistance against bacterial blight using FISH and BAC clones, whereas Asghar et al. (1998) applied FISH for detecting chromosomes of *O. sativa* and *O. officinalis* on the basis of rDNA loci on somatic chromosomes of their hybrid. Koumbaris and Bass (2003) used maize chromosome 9 addition line and an improved single locus FISH protocol to localize loci on pachytene chromosomes. FISH using ribosomal probes of alien species (*Pisum fulvum*) was carried out to confirm the presence of alien chromosome segments in genome of hybrids *P. sativum* × *P. fulvum* and the segregation of these alien chromosomes in the selfed and backcross generations (De Martino et al. 2000).

The powerful technique of FISH can also be used to visualize transgene integration sites and provides a better understanding of transgene behaviour. Studies using FISH to characterize transgene integration have focused primarily on metaphase chromosomes, because the number and position of integration sites on the chromosomes are more easily determined at this stage. However, native or alien gene expression occurs mainly during interphase (Santos et al. 2006). The transgene in *Agrobacterium*-mediated *Cry1A*(*b*)-transgenic rice plants has been detected and its chromosomal location was determined by FISH. Eight of the nine transgenic lines tested showed hybridization signals and demonstrated that *Agrobacterium*-mediated genes can integrate into multiple sites distributed in different parts of the chromosome. The distal regions were reported to be the preferred sites, whereas regions near the centromeres were suggested as the nonpreferential sites for T-DNA integration. Although, whatever the site may be but the transformed DNA sequences remained linked in the recipient genome (Jin et al. 2002). Similarly, fertile transgenic barley was evaluated and FISH revealed that transgene has been integrated in seven out of 19 lines at distal position, whereas four at telomeric region, three each at centromeric and satellite regions and two in subtelomeric regions (Choi et al. 2002).

The ease with which FISH can rapidly and accurately localize the alien introgressions that account for desirable traits from alien species and its broad

applications in structural, comparative, and functional genomics, place this plant molecular cytogenetic tool in a unique position to complement, accelerate, or guide plant-genome research. ISH can also be carried out using total genomic DNA (GISH), chromosome-derived DNA probes, or large genomic insert clones such as bacterial artificial chromosomes (BAC-FISH) (Anamthawat-Jonsson 2001; Schubert et al. 2001; Raina and Rani 2001; Herrera et al. 2007) as several modifications of FISH.

8.4.3 Genomic In Situ Hybridization (GISH)

GISH is a genomic painting technique which allows parental genomes in interspecific hybrids to be distinguished. Total genomic DNA from one parent is labelled as a probe and unlabelled total DNA of the other parent is used as a block. Alternatively, total DNA from both the parents may be labelled and these are both used as probes, each one labelled with a different fluorochrome. This technique is based on the rapid evolution during speciation of repeated sequences, which represents the major part of plant DNA. If the species are distant enough, the repeat sequences allow the chromosomes from the two parental species to be differentiated. The concept of GISH (a modified FISH) was given by Schwarzacher et al. (1989) and was first used in *Hordeum chilense* × *Secale africanum* and *Triticum aestivum* × *Secale cereale*. The advantage of GISH is the recognition of all alien chromosome segments contained in the nucleus and is therefore the method of choice when interspecific crosses and derived introgressed lines are analyzed to reveal alien chromosomes and translocations (Mukai 1994, 1995b; Mukai et al. 1993a; Schwarzacher et al. 2011). GISH is used for identification of chromosomes belonging to different genomes in a polyploid species or for physical localizations of alien introgressions (Heslop-Harrison et al. 1999). This technique also allows visualization of chromosome pairing in interspecific hybrids which determines the production of recombinations or translocations (Kopecky et al. 2009).

Ribeiro-Carvalho et al. (1997) detected spontaneously introgressed rye chromosome segments in a wheat landrace, Barbela using genomic DNA of rye as probe and later, the introgressions were allocated onto wheat chromosome arm 2DL in two of the lines (Ribeiro-Carvalho et al. 2001). Likewise, various workers have identified rye chromosomes (Forsstrom et al. 2002; Chaudhary and Mukai 2004) or wheat–rye translocations like 7BS/7RL (Carvalho et al. 2009), 1BL/1RS (Fig. 8.1), and 6BL/1RS (Yan et al. 2009; Chaudhary 2004; Jeberson 2010). Total rye genomic DNA was utilized by Lima-Brito et al. (1996) for detection of rye genome in triticale × tritordeum F_1 hybrids. Szarka et al. (2002) used GISH for verification of the maize + wheat somatic hybrids. GISH can also be used to detect presence of the chromosomes of different species in an intergeneric cross like wheat × *Imperata cylindrica* (Chaudhary et al. 2013) where the chromosome of two species can be seen distinctly in two different colours to verify the wide hybridization (Fig. 8.2). Similarly, GISH of selected maize-carrying somatic hybrid regenerants revealed

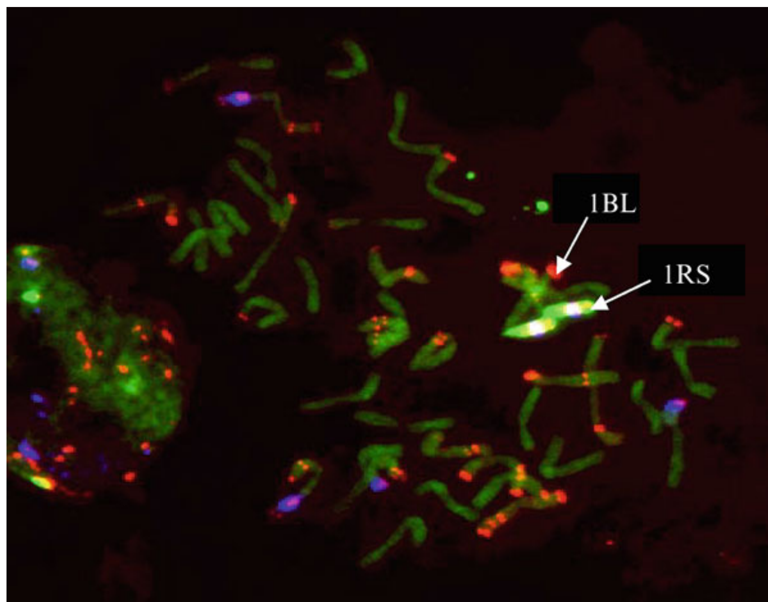

Fig. 8.1 FISH on metaphase spreads of triticale × wheat derived DH line of bread wheat revealing 1BL/1RS translocation (Source: Chaudhary 2004)

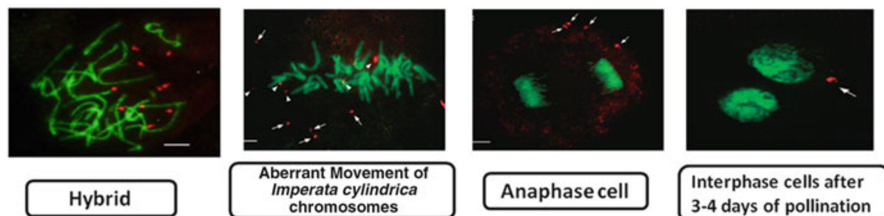

Fig. 8.2 Confirmation of hybrid nature of wheat × *I. cylindrica* derived zygotes and the sequential elimination of *I. cylindrica* chromosomes during first zygotic mitosis (Source: Komeda et al. 2007)

maize chromatin dispersed throughout the wheat nuclear genome by Xu et al. (2003). Likewise, GISH was used effectively to identify *Haynaldia villosa* chromosomes (Zhong et al. 1996), small translocations of wheat grass (Han et al. 1998), *Elymus rectisetus* (Wang et al. 2006), and *Aegilops speltoides* (Klindworth et al. 2012) chromosomes in the genetic background of bread wheat. Li et al. (2000) used GISH to characterize single and reciprocal chromosome translocations arising from tissue culture in hybrids of *T. aestivum* × *T durum-Dassypyrum villosum* amphiploid. GISH of *T. durum* × *Aegilops squarrosa* L. indicated that the translocated chromosome 1A/1D had a terminal 1DL segment of about 35–40 % of the recombinant arm length (Blanco et al. 2002). Zhang et al. (2002) developed seven wheat-*Thinopyrum bessarabicum* disomic addition lines. They characterized them on the

basis of GISH and reported that six of the seven addition lines were true disomic addition lines, whereas one was a duplication–translocation addition line. A BC_1 population from the cross of Tal-Ph^I/*Triticum durum-Dasypyrum villosum* amphiploid (AABBVV)//CS-Ph^I, and its progenies were screened for chromosome recombination by GISH analysis and a homozygous translocation line T5VS.5VL-5DL was identified by Li et al. (2011).

Such introgressions are known to impart resistance against many pests and diseases and harbour traits of agronomic value. Resistance against hessian fly was transferred into breadwheat from resistant durum wheat line by Friebe et al. (1999) and the segments introgressed from alien source were identified using GISH. Similarly, Li et al. (2007) developed five disomic addition lines in wheat, one 5RS ditelosomic addition, two multiple addition lines that confer resistance against wheat spindle streak mosaic virus and the different addition lines were identified using GISH, whereas Fu et al. (2010) utilized GISH to identify the small rye chromatin introgressed into terminal chromosomes of wheat which accounted for improved disease resistance in the translocation lines. A partial amphidiploid line derived from wheat × *Th. intermedium* which was resistant to both powdery mildew and stripe rust was characterized by Bao et al. (2009) using GISH and suggested that resistance is imparted as a result of two St-genome, eight Js-genome, two SATJ chromosomes, and two J-St translocations. GISH of partial wheat-*Th. ponticum* amphidiploids was carried out to identify the physical location of chromosome segment of *Th. ponticum* in translocated chromosome imparting resistance against Fusarium head blight in wheat (Cai et al. 2008). Kang et al. (2011) suggested that GISH has effectively tagged the alien chromosomes harbouring resistance to wheat stripe rust in wheat—*Psathyrostachys huashanica* addition lines. Similarly, four recombinant lines having *Sr39* genes conferring resistance against stem rust with minimal amount of goat grass chromatin were verified using this technique by Niu et al. (2011). Likewise, Bao et al. (2012) used GISH to identify the chromosome segments of *Lymus mollis* harbouring single dominant *Yr* gene for rust resistance in wheat. Zheng et al. (2006) carried out GISH for physical localization of *Th. ponticum* chromosome that imparts blue colour to aleurone in translocated lines of wheat × *Th. ponticum*. The technique of painting chromosomes with genomic probes is useful in detection of parental genomes in interspecific hybrids, identification of alien chromosomes in monosomic alien addition lines (MAALs), and localization of introgressed segments in crop species with chromosomes of small size like rice and wheat. GISH has been used to identify alien introgression in interspecific hybrids of rice with species having different genomes *viz*., *O. officianlis* (CC), *O. brachyantha* (FF), *O. australiensis* (EE), *O. granulata*, and *O. ridleyi* (HHJJ) by Brar and Khush (1997). Yan et al. (1999) used FISH and GISH to identify the chromosomes of *O. eichingeri* in genetic background of rice in F_1, F_2, and back cross generations. Abbasi et al. (1999) used GISH to understand allo- and auto-syntenic pairing among A and E genomes of *O. sativa* and *O. australiensis*, respectively. Similar study was carried out by Hue (2004) among rice genome and C-, BC-, E-, F-, G-, and HJ-genomes. Likewise, in maize GISH helped in demonstrating that small chromosome segments of *Zea mays* ssp. *mexicana* had been integrated into

the maize genome (Wang et al. 2008). Similarly, intergenomic A/D-C translocations were detected using GISH in hexaploid oats and to detect maize chromosomes in oat × maize partial hybrids (Riera-Lizarazu et al. 1996). Ochatt et al. (2004) confirmed the hybrid origin of plants obtained from *P. sativum* × *P. fulvum* cross using GISH which resulted in a clear discrimination of the two parental genomes, using the total genomic DNA probe from *P. fulvum*. F_1 hybrid exhibited seven chromosomes each from *P. sativum* and seven from *P. fulvum*. Application of GISH in advanced generations indicated translocation events taking place between two parental genomes. Fahleson et al. (1997) performed GISH using differently labelled total DNA from the two parental species, in combination with a preannealing step to remove common sequences. The labelling in *Brassica napus* was restricted to the centromeric regions, while a uniform distribution over the chromosomes was found in *Eruca sativa*. GISH revealed that the somatic hybrid progeny contained one or two complete *E. sativa* chromosome(s) but no intergenomic translocation could be detected. Similarly, the alien chromosomes belonging to different genomes in *Brassica* amphidiploids and chromatin of *Rhaphanus sativus* were detected using GISH by Snowdon et al. (1997) and in *Brassica rapa* × *Isatis indigotica* by Tu et al. (2009). Jacobsen et al. (1994) and Garriga-Caldere et al. (1997) detected alien chromosomes of tomato in potato–tomato fusion hybrid by using GISH. Likewise, Dong et al. (2005) also identified chromosomes of *Solanum brevidens* in potato-*S. brevidens* hybrids. Unilateral and reciprocal translocations were detected using GISH among S and T genome of amphidiploids *Nicotiana tabaccum*. Desel et al. (2002) demonstrated GISH as useful tool for high resolution detection of translocations resultant of interspecific hybridization which are only 1 mb in size in genus *Beta*. According to Harper et al. (2011), alien introgressions can be detected by GISH in *Lolium-Festuca* interspecific hybrids and assigned to chromosome arms to create a physical map and suggested that genes of interest may then be located more accurately following further recombination events which reduce the size of the relevant alien introgression. Herrera et al. (2007) used GISH to identify alien chromatin segments on chromosome spreads of *Caffea liberica* introgressed from *C. arabica*. The introgressed fragment carried the SH_3 factor involved in resistance to the coffee rust. Liu et al. (2009) reported that GISH could be successfully used to identify alien chromosome or chromosome segments during interspecific gene transfer in sunflower.

In totality, GISH approach of molecular cytogenetics has been reported as a novel tool not only for detection of alien chromatin which may or may not carry the useful genes but has also proved to be helpful in understanding the genomic relationships among diploid and polyploid species.

8.4.4 Multicolour FISH (MFISH)

MFISH involves simultaneous use of more than one fluorescent dye in one experiment to detect multiple loci in a single cytological preparation. MFISH was

first of all carried by Mukai et al. (1993b) for identification of chromosomes belonging to different genomes in a hexaploid wheat. These researchers used as much as seven differentially labelled probes showing seven different colours. Later, MGISH was used to identify wheat–rye hybrids or translocation lines using mixture of probes corresponding to A, B, D-, and R-genomes (Sanchez-Moran et al. 1999; Hasterok et al. 2002; Han et al. 2003, 2004; Chaudhary 2004; Schwarzacher et al. 2011; Chaudhary et al. 2013; Chaudhary 2013). Castilho et al. (1996) carried out multicolour FISH to identify the breakpoints in translocated chromosomes in wheat-*Ae*. *umbellulata* recombinant lines and Biagetti et al. (1999) in wheat-*Ae*. *longissima* powdery mildew resistant recombinant lines. Vidal et al. (2005) used multicolour FISH to identify instability of *Th*. *ponticum* amphiploids and reported that only cells with 2n = 56 had entire genome of wheat along with two monoploid chromosome sets of *Th*. *ponticum*. Lima-Brito et al. (2006) confirmed hybrid nature of wheat × tritodeum F_1 hybrids by using genomic DNA from *Hordeum chilense* and ribosomal segments of pTa71. Lang Molnar et al. (2006) used MFISH with GAA, pAs1, Hv701, Afa family, pTa 71, and genomic probes for identification of disomic and monosomic wheat/barley addition lines. Sepsi et al. (2008) utilized MFISH to identify 16 chromosomes originating from *Th*. *ponticum*, 14 from A genome, 14 from B genome, and 12 from D genome in wheat–*Th*. *ponticum* hybrids using differentially labelled genomic probes. Also the rearrangements of these chromosomes were visualized in advanced generations of the hybrid. Szakacs and Molnar-Lang (2010) checked genetic stability of disomic wheat–rye addition lines using FISH based on repetitive DNA probes pSc 119.2 and (ACC)5 as well as rDNA probes (5S and 45 S) FISH revealed the chromosome rearrangements. Tang et al. (2005) confirmed successful introgression of three chromosomes of *Z*. *perennis* into a maize-perennial teosinte substitution line.

Conclusively, MFISH provided an edge over the earlier techniques of molecular cytogenetics by utilizing combination of probes for targeting more than one site and hence overcoming the limitations of radioactive ISH.

8.4.5 Nuclei FISH

When interphase nuclei are subjected to FISH analysis, that technique is termed as nuclei FISH or interphase FISH. The advantage of nuclei FISH is that it can be applied to cells from any tissue and at any stage of cell division and cells are not required to be in metaphase stage. It is also beneficial in the study of samples where low mitotic index is observed. Interphase FISH is also useful to understand the organization of chromatin fibres and centromeric regions or telomeric regions during interphase. Leitch et al. (1990) carried out GISH in interphase nuclei of *Hordeum chilense* × *Secale africanum* for physical localization of chromatin of different genomes during interphase nuclei and reported that the genomes of both the species remain at different domains. Similarly, Kosina and Heslop-Harrison (1996) used GISH to analyze lines derived from an amphidiploid between tetraploid wheat (*T*. *durum*) and wheat grass.

Chromosomal aberrations and translocations from similar genomes of J and E into A and B genomes of tetraploid wheat were reported and using interphase FISH they revealed that the three genomes were not intermixed and often lay in distinct sectors. Angelova and Georgiev (2006) reported like other workers that the genetic material of different species in interspecific hybrids remain separate during interphase. Georgiev (2008) reported that FISH in interphase nuclei helped in identification of active rRNA genes from rye chromosomes in translocation mutants of wheat. Apart from physical localization of different genomes, interphase FISH has also helped in identifying the number of alien chromosomes or small segments introgressed into target genome (Wetzel and Rayburn 2000). Detection of the wild beet chromosome by using FISH with the satellite repeat pTS5 and 18S–5.8S–25S rRNA genes in *Beta vulgaris* monosomic addition lines in interphase nuclei (apart from FISH and GISH on metaphase chromosomes) was done by Schmidt et al. (1997).

The nuclei FISH, hence, is an potential tool for understanding the behaviour of two different genomes when brought together via wide hybridization. It also helps in detecting the number and presence of active genes in the genome.

8.4.6 Fibre FISH or Extended DNA Fibre (EDF) Map

The organization of gene and DNA structures can be visualized by ISH of probes to DNA fibres extended to their full molecular length. The technique was first of all given by Fransz et al. (1996). Theoretical consideration of the length of the extended DNA molecule and calibration from hybridization with probes of known length and interspersion pattern (Fransz et al. 1996; Sjöberg et al. 1997; Brandes et al. 1997) can relate the lengths of observed fibres to the numbers of bases. EDF provides an alternative to interphase or metaphase preparations. In this approach, interphase chromosomes are attached to a slide and stretched out in a straight line (rather than being tightly coiled, as in conventional FISH, or adopting a random conformation, as in interphase FISH). The preparation of fibre FISH samples is a skilled art and only specialized laboratories use the technique routinely. The basis of fibre FISH is to release DNA-molecules from the nucleus and to fix the stretched DNA in a linear fashion (parallel to each other) onto a glass slide. The DNA fibres are hybridized with a set of probes labelled with different fluorochromes that cover the entire region of interest resulting in a characteristic string of FISH signals that have the attributes of a multicolor barcode. Barcodes up to 1,000 kb can be generated but a length of approx 300 kb is used in practice. When larger regions are targeted, artificial breakage of the target DNA may complicate interpretation of results.

There are two methods for preparation of extended DNA fibres. One is mechanical shearing along the length of the slide (either to cells that have been fixed to the slide and then lysed or to a solution of purified DNA). The other method is chromosome combing in which the extended conformation of the chromosomes allows dramatically higher resolution, even down to a few kilobases. This method is used to prepare parallel extended DNA fibres. EDF is capable of identification of a DNA

sequence as short as 10–20 kb, whereas in a metaphase FISH a sequence of about 100 kb and above can be detected. The use of fibre FISH is restricted on a larger scale for physical localization of unique or repetitive sequences and only to a limited scale for identification of alien chromatins, but it is quiet efficient for detection of transgenes. The hybridization of *Agrobacterium* T-DNA sequences in transgenic potato plants to extended DNA fibres revealed that T-DNA copies are closely integrated. Moreover, by using probes to T-DNA and vector sequences the composition and arrangement of inserts can be assessed (Wolters et al. 1998). Similarly, relatively little is known about how transgenes are physically integrated into the host genome by biolistic methods. Using FISH on extended DNA fibres (fibre-FISH), 13 independent transgenic wheat lines were analyzed to determine the structural arrangement of stably inherited transgenes in host-plant chromosomes by Jackson et al. (2001) and three basic integration patterns were observed viz., large tandemly repeated integration, large tandem integrations interspersed with unknown DNA and small insertions, possibly interspersed with unknown DNA, while metaphase FISH showed that the integration of transgenes was in both hetero- and euchromatic, as well as proximal, interstitial and distal, regions of the chromosomes. Stupar et al. (2001) utilized EDF for detection of mitochondrial DNA (mtDNA) fragment insertion into a pericentric region on chromosome 2, and EDF revealed that the mitochondrial DNA content of the nuclei is approximately 2.3 times greater than estimated by contig assembly and subsequent sequence analysis. Physical localization of 92-kb DNA fragment of the wheat *Ha-locus* region introgressed into rice by *Agrobacterium*-mediated transformation was done by FISH on extended DNA fibres. Rearrangements of the large-insert T-DNA, involving duplication, deletion, and insertion, were observed in all four lines (Nakano et al. 2005). FISH on extended DNA fibres (fibre FISH) was performed by Imazawa et al. (2009) on transgenic tobacco plants harbouring multiple 37-kb T-DNA constructs. Five and seven types of integrations were successfully visualized in two transgenic lines. Most of the loci suffered duplication, deletion, and/or translocation, indicating the complex integration events of the medium-size T-DNA. They concluded that fibre-FISH analysis is a powerful tool to analyze organization of multiple T-DNA loci in detail.

With the high and wide ranging resolution (1–1,000 kb) as well as its colour barcoding capacity of fibre FISH, it has proven to be well suited as an adjunct physical mapping tool and for analysis of length polymorphism as well as a valuable tool in molecular pathological research to pinpoint regions of chromosome (DNA) rearrangements at a much larger genomic scope than polymerase chain reaction (PCR), Southern blotting, and interphase-FISH.

8.4.7 Flow Cytometry

Flow cytometry refers to the analysis of moving particles (chromosomes) in a suspension with the help of flow cytometer. Chromosomes are excited by a source of

light (UV or laser) which in turn emit an epi-fluorescence which is filtered through a series of dichroic mirrors. The emitted signals are converted by the in-built programme of the equipment into a graph plotting the intensity of the epi-fluorescence emitted against the count of cells emitting it at a given time.

Wetzel and Rayburn (2000) carried out FISH, GISH, and flow cytometry of two alien addition and one normal wheat lines and lines with larger DNA content were observed to have alien DNA present and the DNA difference accounted to the percent of DNA that is in addition to the replaced DNA of wheat. They suggested that determination of nuclear DNA content via flow cytometry can give the idea whether alien chromosome has been introgressed or not. Ochatt et al. (2004) reported that flow cytometry can be used to confirm the hybrid origin of plants followed by hybridization. The hybrids of *P. sativum* × *P. fulvum* had intermediate 2C & 4C peaks of hybrids in comparison to the parents. Shigyo et al. (2003) used flow cytometry along with GISH to detect the alien chromosomes of *Allium cepa* in disomic alien addition lines of *Allium fistulosum*. Flow cytometry analysis clearly helped in revealing that a double dose of the alien 8A chromosome existed in the addition line that caused fluorescence intensity values spurring in DNA content. Later, Hang et al. (2004) also suggested that flow cytometry can detect variable number of alien chromosomes in the genetic background of *A. cepa* addition lines. Lysak et al. (1999) also suggested that flow cytometry can be used to identify chromosomal translocations also.

The wheat/rye translocation 1BL.1RS is present in many modern wheat cultivars and its presence generates a diagnostic change in flow karyotype. However, the discriminating peak was not sufficiently resolved to allow effective sorting of the translocated chromosome. Other translocation chromosomes especially, 5BL.7BL and 4AL.4AS-5BL produced flow karyotype peaks which were discrete enough to permit sorting. However, the most important breakthrough was that almost all the wheat telosomes (with the exception of 3BL and 5BL), which are maintained in stable cytogenetic stocks covering most of the 42 chromosome arms, could be recognized against the background of the rest of the genome, and sorted. The 3BL and 5BL arms were isolated from stocks carrying them as isochromosomes (Doelzel et al. 2007). The oat × maize chromosome 9 addition line produced a flow peak corresponding to maize chromosome 9 and the chromosome was sorted at a level of purity exceeding 90 % (Li and Arumuganathan 2001).

Although flow cytometry does not allow physical localization of alien chromosomes/chromatin, however such introgressions and translocations could be detected through flow peaks. Hence, allowing rapid detection of plants with alien introgressions.

Likewise, other molecular cytogenetic approaches viz., micro-FISH where whole chromosome, a marker or a particular chromosome band scrapped from metaphase spreads using a micromanipulator is used as a probe after amplification through polymerase chain reaction; primed in situ labelling (PRINS) in which short oligonucleotide primers are annealed to target sequences in situ, followed by elongation of the sequences with a *Taq* polymerase and simultaneous labeling of the target sequences with a fluorochrome and spectral karyotyping (SKY) where a

probe mixture composed of chromosome-specific libraries generated from flow-sorted chromosomes are simultaneously visualized in different colours by means of digital classified spectra. These techniques have emerged as potential tools for enhancing efficiency of crop improvement programmes through precise detection of alien introgressions.

8.5 Conclusion

Introgressive hybridization has become very important in the modern days of climate change to develop cultivars resistant to the prevailing and potential biotic and abiotic stresses. Through this aspect of plant breeding, an enormous amount of genetic variation is being created that will be quite useful for selection and development of improved cultivars. During integration of alien genetic material in crop plants, the characterization of the introgressed genes is very essential in order to confirm their transfer into the genetic background of recipient crop plants with minimum linkage drag. During the past years, it has become obvious that genetic and physical maps are not directly comparable as chiasmata are not evenly distributed along the chromosome axis. Molecular cytogenetic tools viz., fluorescence in situ hybridization (FISH), genomic in situ *hybridization* (GISH), multicolour FISH, fibre FISH, spectral karyotyping (SKY), and primed in situ hybridization (PRINS) have shown a great promise in terms of proper identification and localization of the alien chromosome segments in the genetic background of crop varieties. By integrating physical maps created by GISH and genetic linkage maps, the precise site of genes on a chromosome arm may be determined and resulting markers tightly linked to the genes of interest can be used for future breeding programmes (Humphreys et al. 1998). As compared to in situ hybridization, FISH and GISH have increased the effectiveness of the introgressive hybridization programmes through high resolution mapping of the targeted alien chromosome segments. Multicolour FISH, a modification of FISH has provided the ways to characterize the introgressed chromosome segments of more than one species simultaneously. The resolution of FISH has been increased to a great extent through extended DNA fibre mapping (EDF). Extended DNA fibres allow characterization of the introgressed alien segments beyond the limits of FISH. Similarly, other techniques like SKY, PRINS, micro-FISH, and CGM have also increased the efficiency of alien gene transfer in crop plants.

Conclusively, it can be stated that molecular cytogenetics has become an invaluable and inevitable part of targeted, highly precise and efficient introgressive hybridization undertaken to broaden the genetic base of cultivated crop varieties.

Acknowledgements The authors are highly obliged to Prof. Yasuhiko Mukai, Osaka Kyoiku University, Japan and Dr. Trude Schwarzacher, Department of Biology, University of Leicester, UK for extending their expertise in getting the resolution of certain results mentioned in this article.

References

Abbasi FM, Brar DS, Carpena AL, Fukui K, Khush GS (1999) Detection of autosyndetic and allosyndetic pairing among A and E genomes of *Oryza* through genomic in situ hybridization. Rice Genet Newsl 16:24–25

Ahmad F, Comeau A, Chen Q, Collin J, St Pierre CA (2000) Radiation induced wheat-rye chromosomal translocations in triticale: optimizing the dose using fluorescence in situ hybridization. Cytologia 65(1):1–6

Allard RW (1996) Genetic basis of the evolution of adaptedness in plants. Euphytica 92:1–11

Ambros PP, Matzke MA, Matzke AJM (1986) Detection of a 17 kb unique sequence (T-DNA) in plant chromosomes by in situ hybridization. Chromosoma 34:11–18

Anamthawat-Jonsson K (2001) Molecular cytogenetics of introgression hybridization in plants. Methods Cell Sci 23:139–148

Angelova Z, Georgiev S (2006) Visualization of *Secale cereale* DNA in wheat germplasm by genomic in situ hybridization. Biotechnol Biotechnol Equip 3:26–29

Anuradha C, Mahal GS, Singh S (2006) Alien introgression for quality characters from *T. dicoccum* and *T. dicoccoides* to durum wheat. Crop Improv 33(1):9–15

Armstrong KC, Molnar SJ, Fedak G (1992) Differential transmission of homoeologus chromosomes in heterozygous amphiploids: rRNA loci of *Thinopyrum distichum* in an amphiploid with *Triticum durum*. Genome 35(6):985–991

Asghar M, Brar DS, Hernandez JE, Ohmido N, Khush GS (1998) Characterization of parental genomes in a hybrid between *Oryza sativa* L. and *O. officinallis* Wall ex Watt. through fluorescence in situ hybridization. Rice Genet Newsl 15:83–84

Bao Y, Li X, Liu S, Cui F, Wang H (2009) Molecular cytogenetic characterization of a new wheat-*Thinopyrum intermedium* partial amphiploid resistant to powdery mildew and stripe rust. Cytogenet Genome Res 126:390–395

Bao Y, Wang J, He F, Ma H, Wang H (2012) Molecular cytogenetic identification of a wheat (*Triticum aestivum*)-American dune grass (*Leymus mollis*) translocation line resistant to stripe rust. Genet Mol Res 11:3198–3206

Biagetti M, Vitellozzi F, Ceoloni C (1999) Physical mapping of wheat–*Aegilops longissima* breakpoints in mildew-resistant recombinant lines using FISH with highly repeated and low-copy DNA probes. Genome 42:1013–1019

Blanco A, Cenci A, Simeone R, Gadaleta A, Pignone D, Galasso I (2002) The cytogenetics and molecular characteristics of a translocated chromosome 1AS.1AL-1DL with a *Glu-D1* locus in durum wheat. Cell Mol Biol 7:559–567

Bostock CJ, Sumner AT (1978) The eukaryotic chromosome. North Holland Publishing Company, Amsterdam, p 525

Brandes A, Thompson H, Dean C, Heslop-Harrison JS (1997) Multiple repetitive DNA sequences in the paracentromeric regions of *Arabidopsis thaliana* L. Chromosome Res 5:238–246

Brar DS, Khush GS (1997) Alien introgression in rice. Plant Mol Biol 35(1–2):35–47

Brar DS, Khush GS (2005) Cytogenetic manipulation and germplasm enhancement of rice (*Oryza sativa* L). In: Singh RJ, Jauhar PP (eds) Genetic resources, chromosome engineering, and crop improvement series: cereals. CRC Press, Boca Raton, FL, pp 115–158

Brown GG, Formanova N, Jin H, Wargachuk R, Dendy C, Patil P, Laforest M, Zhang J, Cheung WY, Landry BS (2003) The radish *Rfo* restorer gene of *Ogura* cytoplasmic male sterility encodes a protein with multiple pentatricopeptide repeats. Plant J 35:262–272

Buongiorno-Nardelli M, Amaldi F (1969) Autoradiographic detection of molecular hybrids between rRNA and DNA in tissue sections. Nature (London) 225:946–947

Cai X, Xu SS, Oliver RE, Zhang Q, Stack RW, Zhong S, Friesen TL, Halley S, Elias EM (2008) Alien introgression for FHB resistance in wheat—challenges and strategies. In: Appels R et al (eds) Proceedings of 11th international wheat genetics symposium. Sydeny University Press, Australia, pp 716–718

Carvalho A, Martin A, Heslop-Harrison JS, Guedes-Pinto H, Lima-Brito J (2009) Identification of the spontaneous 7BS/7RL intergenomic translocation in one F_1 multigeneric hybrid from the Triticeae tribe. Plant Breed 128:105–108

Casperson T, Lomakka G, Moller A (1971) Computerized chromosome identification by aid of the quinacrine mustard fluorescence technique. Heretitas 67:103–110

Castilho A, Miller TE, Heslop-Harrison JS (1996) Physical mapping of translocation breakpoints in a set of wheat-*Aegilops umbellulata* recombinant lines using in situ hybridization. Theor Appl Genet 93(5–6):816–825

Ceoloni C, Forte P, Gennaro A, Micali S, Carozza R, Bitti A (2005) Recent developments in durum wheat chromosome engineering. Cytogenet Genome Res 109:328–334

Chan SWL (2010) Chromosome engineering: power tools for plant genetics. Trends Biotechnol 28:605–610

Chaudhary HK (2004) Molecular cytogenetic analysis of rye introgressed triticale × wheat derivatives. Report submitted to Commonwealth Scholarships Commission. UK, London, p 6

Chaudhary HK (2013) New frontiers in chromosome elimination-mediated doubled haploidy breeding for accelerated and high precision genetic upgradation in wheat. In: Plant and animal genome meeting in the International Triticeae Mapping Initiative workshop. Cornell University, USA

Chaudhary HK, Mukai Y (2004) Molecular cytogenetic detection of rye introgressed wheat recombinants. Report of Sr. Biotechnology Overseas Associatship, DBT, GOI. New Delhi, India, p 25

Chaudhary HK, Tayeng T, Kaila V, Rather SA (2013) Enhancing the efficiency of wide hybridization mediated chromosome engineering for high precision crop improvement with special reference to wheat × *Imperata cylindrica* system. Nucleus. doi:10.1007/s13237-013-0077-5

Chen G, Zheng Q, Bao Y, Liu S, Wang H, Li X (2012) Molecular cytogenetic identification of a novel dwarf wheat line with introgressed *Thinopyrum ponticum* chromatin. J Biosci 37:149–155

Choi HW, Lemaux PG, Cho MJ (2002) Use of fluorescence in situ hybridization for gross mapping of transgenes and screening for homozygous plants in transgenic barley (*Hordeum vulgare* L). Theor Appl Genet 106:92–100

Darlington CD, La Cour LF (1969) The handling of chromosomes, 5th edn. G. Allen, London

De Martino T, Errico A, Lassandro A, Conicella C (2000) Distorted segregation resulting from pea chromosome reconstructions with alien segments from *Pisum fulvum*. Am Genet Assoc 91: 322–325

Desel C, Jansen R, De Dong G, Schmidt T (2002) Painting of parental chromatin in *Beta* hybrids by multi-colour fluorescent in situ hybridization. Ann Bot 89(2):171–181

Desloire S, Gherbi H, Laloui W, Marhadour S, Clouet V, Cattolico L, Falentin C, Giancola S, Renard M, Budar F, Small I, Caboche M, Delourme R, Bendahmane A (2003) Identification of the fertility restoration locus, *Rfo*, in radish, as a member of the pentatricopeptide-repeat protein family. EMBO Rep 4:588–594

Doelzel J, Kubalakova M, Paux E, Bartos J, Feuillet C (2007) Chromosome based genomics in the cereals. Chromosome Res 15:51–66

Dong F, Tek AL, Frasca ABL, McGrath JM, Wielgus SM, Helgeson JP, Jiang J (2005) Development and characterization of potato-*Solanum brevidens* chromosomal addition/substitution lines. Cytogenet Genome Res 109:368–372

Donini P, Koebner RMD, Ceoloni C (1995) Cytogenetic and molecular mapping of the wheat-*Aegilops longissima* chromatin breakpoints in powdery mildew resistant introgression lines. Theor Appl Genet 91:738–743

Dyer AF (1963) The use of lacto-propionic orcein in rapid squash methods for chromosome preparations. Stain Technol 38:85–90

Fahleson J, Lagercrantz U, Mourasb A, Glimelius K (1997) Characterization of somatic hybrids between *Brassica napus* and *Eruca sativa* using species-specific repetitive sequences and genomic in situ hybridization. Plant Sci 123:133–142

Forsstrom PO, Merker A, Schwarzacher T (2002) Characterization of mildew resistant wheat-rye substitution lines and identification of an inverted chromosome by fluorescent in situ hybridization. Heredity 88(5):349–355

Fransz PF, Alonso–Blanco C, Liharska TB, Peeters AJM, Zabel P, de Jong JH (1996) High-resolution physical mapping in *Arabidopsis thaliana* and tomato by fluorescence in situ hybridization to extended DNA fibres. Plant J 9:421–430

Friebe B, Kynast RG, Hatchett JH, Sears RG, Wilson DL, Gill BS (1999) Transfer of wheat-rye translocation chromosomes conferring resistance to hessian fly from bread wheat into durum wheat. Crop Sci 39:1692–1696

Fu S, Tang Z, Ren Z, Zhang H (2010) Transfer to wheat (*Triticum aestivum*) of small chromosome segments from rye (*Secale cereale*) carrying disease resistance genes. J Appl Genet 51(2): 115–121

Gall JG, Pardue ML (1969) Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proc Natl Acad Sci U S A 63:378–383

Garriga-Caldere F, Huigen DJ, Filotico F, Jacobsen E, Ramanna MS (1997) Identification of alien chromosomes through GISH and RFLP analysis and the potential for establishing potato lines with monosomic additions of tomato chromosomes. Genome 40(5):666–673

Georgiev S (2008) Activity of rRNA genes from rye chromosome in translocation mutant forms of *T. aestivum* L. Biotechnol Biotechnol Equip 2:687–690

Gill BS, Friebe B (1998) Plant cytogenetics at the dawn of the 21st century. Curr Opin Plant Biol 1:109–115

Gill BS, Kimber G (1974) Giemsa C-banding and the evolution of wheat. Proc Natl Acad Sci U S A 71:4086–4090

Han FP, He MY, Bu XL, Huang BQ, Hao S, Ma YZ, Xin ZY (1998) Characterization of a wheat-wheat grass translocation line by FISH. Acta Botanica Sinica 40(6):500–502

Han F, Fedak G, Benabdelmouna A, Armstrong K, Ouellet T (2003) Characterization of six wheat × *Thinopyrum intermedium* derivatives by GISH, RFLP and multicolor GISH. Genome 46:490–495

Han F, Liu B, Fedak G, Liu Z (2004) Genomic constitution and variation in five partial amphiploids of wheat–*Thinopyrum intermedium* as revealed by GISH, multicolor GISH and seed storage protein analysis. Theor Appl Genet 109:1070–1076

Hang TTM, Shigyo M, Yamauchi N, Tashiro Y (2004) Production and characterization of alien chromosome additions in shallot (*Allium cepa* L. Aggregatum group) carrying extra chromosomes of Japanese bunching onion (*A. fistulosum* L.). Genes Genet Syst 79:263–269

Harper J, Armstead I, Thomas A, James C, Gasior D, Bisaga M, Roberts L, King I, King J (2011) Alien introgression in the grasses *Lolium perenne* (perennial ryegrass) and *Festuca pratensis* (meadow fescue): the development of seven monosomic substitution lines and their molecular and cytological characterization. Ann Bot 107(8):1313–1321

Harper LC, Cande WZ (2000) Mapping a new frontier: development of integrated cytogenetic maps in plants. Funct Integr Genomics 1:89–98

Hasterok R, Langdon T, Taylor S, Jenkins G (2002) Combinatorial labelling of DNA probes enables multicolour fluorescence in situ hybridization in plants. Folia Histochem Cytobiol 40(3):319–323

Herrera JC, D'Hont A, Lashermes P (2007) Use of fluorescence in situ hybridization as a tool for introgression analysis and chromosome identification in coffee (*Coffea arabica* L.). Genome 50:619–626

Heslop-Harrison JS, Osuji J, Hull R, Harper G (1999) Fluorescent in situ hybridization of plant chromosomes: illuminating the Musa genome. In INIBAP annual report 1998. INIBAP, Montpellier (Fra), pp 26–29

Hoisington D, Khairallah M, Reeves T, Ribaut JM, Skovmand B, Taba S, Warburton M (1999) Plant genetic resources: what can they contribute toward increased crop productivity? Proc Natl Acad Sci U S A 96:5937–5943

Hue NTN (2004) Homoeologous chromosome pairing and alien introgression analysis in wide cross derivatives of *Oryza* through fluorescence in situ hybridization. Ph.D. thesis, UPLB, Los Banos, Phillipines, p 160.

Humphreys MW, Pasakinskiene I, James AR, Thomas H (1998) Physically mapping quantitative traits for stress-resistance in the forage grasses. J Exp Bot 49(327):1611–1618
Imazawa T, Suzuki G, Nakano A, Yamamoto M, Mukai Y (2009) Visualization of multiple T-DNA loci by FISH on extended DNA fibres. Plant Biotechnol 26:421–425
Jackson SA, Zhang P, Chen WP, Phillips RL, Friebe B, Muthukrishnan S, Gill BS (2001) High-resolution structural analysis of biolistic transgene integration into the genome of wheat. Theor Appl Genet 103:56–62
Jacobsen E, De Jongi JH, Kamstra SA, Van den berg PMMM, Ramanna MS (1994) Genomic in situ hybridization (GISH) and RFLP analysis for the identification of alien chromosomes in the backcross progeny of potato (+) tomato fusion hybrids. Heredity 74:250–257
Jauhar PP, Peterson TS, Xu SS (2009) Cytogenetic and molecular characterization of a durum alien disomic addition line with enhanced tolerance to Fusarium head blight. Genome 52:467–483
Jeberson S (2010) Physical mapping of some triticale × wheat derived rye chromatin introgressed wheat recombinants through fluorescence in situ hybridization. Ph.D. thesis. CSK HP Agricultural University, Palampur, Himachal Pradesh, India, p 127.
Jellen EN, Leggett JM (2005) Cytogenetic manipulation in oat improvement. In: Singh RJ, Jauhar PP (eds) Genetic resources, chromosome engineering, and crop improvement series: cereals, vol 2. CRC Press, Boca Raton, FL, pp 199–231
Jiang J, Gill BS (1994) Nonisotopic in situ hybridization and plant genome mapping: the first 10 years. Genome 37:717–725
Jiang J, Gill BS, Wang GL, Ronald PC, Ward DC (1995) Metaphase and interphase fluorescence in situ hybridization mapping of the rice genome with bacterial artificial chromosomes. Proc Natl Acad Sci U S A 92:4487–4491
Jin WW, Li ZY, Fang Q, Altosaar I, Liu LH, Song YC (2002) Fluorescence in situ hybridization analysis of alien genes in *Agrobacterium*-mediated *Cry1A*(*b*)-transformed rice. Ann Bot 90:31–36
John H, Birnstiel M, Jones K (1969) RNA–DNA hybrids at the cytological level. Nature (London) 223:582–587
Kang H, Wang Y, Fedak G, Cao W, Zhang H, Fan X, Sha L, Xu L, Zheng Y, Zhou Y (2011) Introgression of chromosome 3Ns from *Psathyrostachys huashanica* into wheat specifying resistance to stripe rust. PLoS One 6(7):e21802
Kannan TP, Zilfalil BA (2009) Cytogenetics: past, present and future. Malays J Med Sci 16(2):4–9
Klindworth DL, Niu Z, Chao S, Friesen TL, Jin Y, Faris JD, Cai X, Xu SS (2012) Introgression and characterization of a goatgrass gene for a high level of resistance to Ug99 stem rust in tetraploid wheat. Genes Genome Genet 2:665–673
Komeda N, Chaudhary HK, Mukai Y (2007) Cytological evidence for chromosome elimination in wheat × *Imperata cylindrica* hybrids. Genes Genet Syst 82:241–248
Kopecky D, Bartos J, Zwierzykowski Z, Dolezel J (2009) Chromosome pairing of individual genomes in tall fescue (*Festuca arundinacea* Schreb.), its progenitors and hybrids with Italian ryegrass (*Lolium multiflorum* Lam.). Cytogenet Genome Res 124(2):170–178
Kosina R, Heslop-Harrison JS (1996) Molecular cytogenetics of an amphiploid trigeneric hybrid between *Triticum durum*, *Thinopyrum distichum* and *Lophopyrum elongatum*. Ann Bot 78: 583–589
Koumbaris GL, Bass HW (2003) A new single-locus cytogenetic mapping system for maize (*Zea mays* L.): overcoming FISH detection limits with marker-selected sorghum (*S. propinquum* L.) BAC clone. Plant J 35:647–659
Lang Molnar M, Szakacs E, Linc G (2006) Identification of newly developed wheat/winter barley addition lines using fluorescence in situ hybridization and SSR markers. Pflanzenzüchtung und Genomanalyse 57:75–77
Lapitan NLV, Sears RG, Rayburn AL, Gill BS (1986) Wheat-rye translocations: detection of chromosome breakpoints by in situ hybridization with a biotin-labeled DNA probe. J Hered 77(6):415–419

Leitch AR, Mosgoller W, Schwarzacher T, Bennett MD, Heslop-Harrison JS (1990) Genomic in situ hybridization to sectioned nuclei shows chromosome domains in grass hybrids. J Cell Sci 95:335–341

Leitch IJ, Leitch AR, Heslop-Harrison JS (1991) Physical mapping of plant DNA sequences by simultanious in situ hybridization of two differently labelled fluorescent probes. Genome 34:329–333

Li AX, Qi ZJ, Pei ZY, Zhuang LF, Feng YG, Wang XE (2007) Development and WSSMV resistance identification of wheat landrace Huixianhong alien chromosome lines derived from rye cultivar Jingzhouheimai. Acta Agronomica Sinica 33(4):639–645

Li HF, Gill BS, Wang X, Chen PD (2011) A *Tal-PhI* wheat genetic stock facilitates efficient alien introgression. Genet Resour Crop Evol 58(5):667–678

Li HJ, Guo BH, Li YW, Du LQ, Jia X, Chu CC (2000) Molecular cytogenetic analysis of intergeneric chromosomal translocations between wheat (*Triticum aestivum* L.) and *Dasypyrum villosum* arising from tissue culture. Genome 43:756–762

Li L, Arumuganathan K (2001) Physical mapping of 45S and 5S rDNA on maize metaphase and sorted chromosomes by FISH. Hereditas 134:141–145

Lima-Brito J, Carvalho A, Martin A, Heslop-Harrison JS, Guedes-Pinto H (2006) Morphological, yield, cytological and molecular characterization of a bread wheat × tritorderm F_1 hybrid. J Genet 85:123–131

Lima-Brito J, Guedes-Pinto H, Heslop-Harrison JS (1996) Molecular cytogenetics analysis of triticale × tritordewum F_1 hybrids. Triticale 5:183–188

Liu Z, Feng J, Jan CC (2009) Genomic in Situ Hybridization (GISH) as a tool to identify chromosomes of parental species in sunflower interspecific hybrids. Proceedings of plant and animal genomes XVII conference. p 196

Lysak MA, Cihalikova J, Kubalakova M, Simkova H, Kunzel G, Dolezel J (1999) Flow karyotyping and sorting of mitotic chromosomes of barley (*Hordeum vulgare* L.). Chromosome Res 7:431–444

Merker A (1979) The breeding behaviour of some rye wheat chromosome substitutions. Hereditas 91:245–255

Mujeeb-Kazi A (1998) Evolutionary relationships and gene transfer in the Triticeae. Proceedings of the third international Triticeae symposium. pp 59–65.

Mujeeb-Kazi A, Islam-Faridi MN, Cortes A (1996) Genome identification in some wheat and alien Triticeae species intergeneric hybrids by fluorescent in situ hybridization. Cytologia 61(3): 307–315

Mukai Y (1994) Molecular cytogenetic analysis of chromosomes by the use of genome-specific DNA. In: Recent advances in plant breeding. Yokendo Ltd, Tokyo, pp 92–97

Mukai Y (1995a) Molecular cytogenetic analysis of plant chromosomes by in situ hybridization. In: Tsunewaki K (Commemoration of Professor Hitoshi Kihara's Contennial) (ed) Plant genome and plastome: their structure and evolution, Kodansha Scientific, Inc, Tokyo

Mukai Y (1995b) Multicolor fluorescence in situ hybridization approach for genome analysis and gene mapping in wheat and its relatives. In: Li ZS, Xin ZY (eds) Proceedings 8th international wheat genetic symposium, pp 543–546.

Mukai Y, Friebe B, Hatchett JH, Yamamoto M, Gill BS (1993a) Molecular cytogenetic analysis of radiation-induced wheat-rye terminal and intercalary chromosomal translocation and the detection of rye chromatin specifying resistance to Hessian fly. Chromosoma 102:88–95

Mukai Y, Nakahara Y, Yamamoto M (1993b) Simultaneous discrimination of the three genomes in hexaploid wheat by multicolor fluorescence in situ hybridization using total genomic and highly repeated DNA probes. Genome 36:489–494

Multani DS, Khush GS, Reyes BGD, Brar DS (2003) Alien genes introgression and development of monosomic alien addition lines from *Oryza latifolia* Desv. to rice, *Oryza sativa* L. Theor Appl Genet 107(3):395–405

Nakano A, Suzuki G, Yamamoto M, Turnbull K, Rahman S, Mukai Y (2005) Integration and organization of large insert T-DNA in rice: visualization of rearrangements of large transgenes. Mol Genet Genomics 273:123–129

Nakata N, Yasumuro Y, Sasaki M (1977) An acetocarmine-Giemsa staining of rye chromosomes. Jpn J Genet 52:315–318

Navabi ZK, Stead KE, Chris Pires J, Xiong Z, Sharpe AG, Parkin IAP, Rahman MH, Good AG (2011) Analysis of B-genome chromosome introgression in interspecific hybrids of *Brassica napus* and *B. carinata*. Genetics 187:659–673

Niu Z, Klindworth DL, Friesen TL, Chao S, Jin Y, Cai X, Xu SS (2011) Targeted introgression of a wheat stem rust resistance gene by DNA marker-assisted chromosome engineering. Genetics 187:1011–1021

Ochatt SJ, Benabdelmouna A, Marget P, Aubert G, Moussy F, Pont'ecaille C, Jacas L (2004) Overcoming hybridization barriers between pea and some of its wild relatives. Euphytica 137: 353–359

Raina SN, Rani V (2001) GISH technology in plant genome research. Methods Cell Sci 23:83–104

Rayburn AL, Gill BS (1985) Use of biotin-labeled probes to map specific DNA sequences on wheat chromosomes. J Hered 76:78–81

Ribeiro-Carvalho C, Guedes-Pinto H, Harrison G, Heslop-Harrison JS (1997) Wheat-rye chromosome translocations involving small terminal and intercalary rye chromosome segments in the Portuguese wheat landrace Barbela. Heredity 78:539–546

Ribeiro-Carvalho C, Guedes-Pinto H, Heslop-Harrison JS, Schwarzacher T (2001) Introgression of rye chromatin on chromosome 2D in the Portuguese wheat landrace "Barbela". Genome 44:1122–1128

Riera-Lizarazu O, Rines HW, Phillips RL (1996) Cytological and molecular characterization of oat × maize partial hybrids. Theor Appl Genet 93:123–135

Rieseberg LH, Baird SJE, Gardner KA (2000) Hybridization, introgression and linkage evolution. Plant Mol Biol 42:205–224

Sanchez-Moran E, Benavente E, Orellana J (1999) Simultaneous identification of A, B, D, and R genomes by genomic in situ hybridization in wheat-rye derivatives. Heredity 83:249–252

Santos AP, Wegel E, Allen GC, Thompson WF, Stoger E, Shaw P, Abranches R (2006) In situ methods to localize transgenes and transcripts in interphase nuclei: a tool for transgenic plant research. Plant Methods 2:18–31

Schmidt T, Jung C, Heslop-Harrison JS, Kleine M (1997) Detection of alien chromatin conferring resistance to the beet cyst nematode (*Heterodera schachtii* Schm.) in cultivated beet (*Beta vulgaris* L.) using in situ hybridization. Chromosome Res 5:186–193

Schubert I, Fransz PF, Fuchs J, Hans de Jong J (2001) Chromosome painting in plants. Methods Cell Sci 23:57–69

Schwarzacher T, Ali N, Chaudhary HK, Graybosch R, Kapalande HV, Kinski E, Heslop-Harrison JS (2011) Fluorescent in situ hybridization as a genetic technology to analyse chromosomal organization of alien wheat recombinant lines. pp 121–129. IAEA TECDOC.

Schwarzacher T, Heslop-Harrison JS (2000) Practical in situ hybridization. Bios., Oxford, pp 203–XII

Schwarzacher T, Leitch AR, Bennett MD, Heslop-Harrison JS (1989) In situ localization of parental genomes in a wide hybrid. Ann Bot 64:315–324

Sepsi A, Molnar I, Szalay D, Molnar-Lang M (2008) Molecular cytogenetic analysis of the wheat-Agropyron *elongatum* partial amphiploid BE-1. Acta Biol Szeg 52(1):139–141

Sethi GS, Plaha P (1988) The nature of rye (*Secale cereale* L.) chromatin introgression into wheat (*Triticum aestivum* L. em. Thell.) via triticale (X *Triticosecale* Whittmack). In: Miller TE, Koebner RMD (eds) Proceedings of the seventh international wheat genetics symposium. Cambridge University Press, Cambridge, pp 433–438

Shigyo M, Wako T, Kojima A, Yamauchi N, Tashiro Y (2003) Transmission of alien chromosomes from selfed progenies of a complete set of *Allium* monosomic additions: the development of a reliable method for the maintenance of a monosomic addition set. Genome 46(6):1098–1103

Simmonds NW (1993) Introgression and incorporation strategies for the use of crop genetic resources. Biol Rev 68:539–562

Singh RJ (2005) Utilization of genetic resources for barley improvement. In: Singh RJ, Jauhar PP (eds) Genetic resources, chromosome engineering, and crop improvement series: cereals, vol 2, CRC Press. Boca Raton, FL, pp 233–255

Sjöberg A, Peelman LJ, Chowdhary BP (1997) Application of three different methods to analyse fibre-FISH results obtained using four lambda clones from the porcine MHCIII region. Chromosome Res 5:247–253

Snow R (1963) Alcoholic hydrochloric acid-carmine as a stain for chromosomes in squash preparations. Stain Technol 38:9–13

Snowdon RJ, Kohler W, Friedt W, Kohler A (1997) Genomic in situ hybridization in *Brassica* amphidiploids and interspecific hybrids. Theor Appl Genet 95:1320–1324

Stupar RM, Lilly JW, Town CD, Cheng Z, Kaul S, Buell CR, Jiang J (2001) Complex mtDNA constitutes an approximate 620-kb insertion on *Arabidopsis thaliana* chromosome 2: implication of potential sequencing errors caused by large-unit repeats. Proc Natl Acad Sci U S A 98(9):5099–5103

Szakacs E, Molnar-Lang M (2010) Molecular cytogenetic evaluation of chromosome instability in *Triticum aestivum–Secale cereal*e disomic addition lines. J Appl Genet 51(2):149–152

Szarka B, Gonter I, Molnar-Lang M, Morocz S, Dudits D (2002) Mixing of maize and wheat genomic DNA by somatic hybridization in regenerated sterile maize plants. Theor Appl Genet 105(1):1–7

Tan FQ, Fu SL, Tang ZX, Ren ZL, Zhang HQ (2009a) Genetic variation of 1RS arm between sibling wheat lines containing 1BL.1RS translocation. J Plant Breed Crop Sci 1(5):204–209

Tan F, Zhou J, Yang Z, Zhang Y, Pan L, Ren Z (2009b) Characterization of a new synthetic wheat-*Aegilops biuncialis* partial amphiploid. Afr J Biotechnol 8(14):3215–3218

Tang Q, Rong T, Song YC, Yang J, Pan G, Li W, Huang Y, Cao M (2005) Introgression of perennial teosinte genome into maize and identification of genomic in situ hybridization and microsatellite markers. Crop Sci 45:717–721

Tanksley SD, McCouch SR (1997) Seed banks and molecular maps: unlocking genetic potential from the wild. Science 277:1063–1066

Tanksley SD, Nelson JC (1996) Advanced backcross QTL analysis: a method for simultaneous discovery and transfer of valuable QTLs from unadapted germplasm into elite breeding lines. Theor Appl Genet 92:191–203

Tu Y, Sun J, Ge X, Li Z (2009) Chromosome elimination, addition and introgression in intertribal partial hybrids between *Brassica rapa* and *Isatis indigotica*. Ann Bot 103:1039–1104

Vidal ACB, Cuadrado A, Brammer SP, Benko-Iseppon AM, Guerra M (2005) Molecular cytogenetic characterization of parental genomes in the partial amphidiploid *Triticum aestivum* × *Thinopyrum ponticum*. Genet Mol Biol 28(2):308–313

Wang CY, Ji WQ, Zhang GS, Wang QY, Xue XZ (2006) Molecular cytogenetics identification on *Triticum aestivum-Elymus rectisetus* alien addition lines. Acta Agronomica Sinica 32(12): 1898–1901

Wang LZ, Yang AF, He CM, Qu ML, Zhang JR (2008) Creation of new maize germplasm using alien introgression from *Zea mays* ssp. *mexicana*. Euphytica 164(3):789–801

Wetzel JB, Rayburn AL (2000) Use of fluorescence genomic in situ hybridization (GISH) to detect the presence of alien chromatin in wheat lines differing in nuclear DNA content. Cytometry 41:36–40

William MDHM, Mujeeb-Kazi A (1995) Biochemical and molecular diagnostics of *Thinopyrum bessarabicum* chromosomes in *Triticum aestivum* germplasm. Theor Appl Genet 90(7–8): 952–956

Wolters AMA, Trindade LM, Jacobsen E, Visser RGF (1998) Fluorescence in situ hybridization on extended DNA fibres as a tool to analyse complex T-DNA loci in Potato. Plant J 13(6): 837–847

Xu C, Xia G, Zhi D, Xiang F, Chen H (2003) Integration of maize nuclear and mitochondrial DNA with the wheat genome through somatic hybridization. Plant Sci 165(5):1001–1008

Yamamoto M, Mukai Y (1989) Application of fluorescence in situ hybridization to molecular cytogenetics of wheat. Wheat Inform Serv 69:30–32

Yan H, Min S, Zhu L (1999) Visualization of *Oryza eichingeri* chromosomes in intergenomic hybrid plants from *O. sativa* × *O. eichingeri* via fluorescent in situ hybridization. Genome 42(1):48–51

Yan L, Wang H, Wu J, Sun DJ, Li XJ, Feng Y, Min DH (2009) Molecular cytogenetics identification of alien chromatin in Xinong 9814. J Northwest A F Univ Nat Sci Edn 37(5):94–98

Yang YP, Chen PD (2009) Molecular cytogenetics, fertility, and scab resistance of the intergeneric hybrid F_1 and BC_1 between *Triticum aestivum* and *Roegneria kamoji*. Hereditas-Beijing 31(3): 290–296

Zhang JY, Li XM, Wang RRC, Cortes A, Rosas V, Mujeeb-Kazi A (2002) Molecular cytogenetic characterization of Eb-genome chromosomes in *Thinopyrum bessarabicum* disomic addition lines of bread wheat. Int J Plant Sci 163(1):167–174

Zheng Q, Li B, Mu S, Zhou H, Li Z (2006) Physical mapping of the blue-grained gene(s) from *Thinopyrum ponticum* by GISH and FISH in a set of translocation lines with different seed colours in wheat. Genome 49:1109–1114

Zhong SB, Zhang DY, Li HB, Yao JX (1996) Identification of *Haynaldia villosa* chromosomes added to wheat using a sequential C-banding and genomic in situ hybridization technique. Theor Appl Genet 92(1):116–120

Zhou XH, Wan HJ, Qian CT, Chen JF (2008) Development and characterization of *Cucumis sativus-hystrix* introgression lines exhibiting resistance to downy mildew. Cucurbitaceae. In: Pitrat M (ed) Proceedings of the IXth EUCARPIA meeting on genetics and breeding of Cucurbitaceae, INRA, Avignon, France

Chapter 9
Agronomically Relevant Traits Transferred to Major Crop Plants by Alien Introgressions

Neeraj Kumar and Sachin Rustgi

Abstract Extensive selection for increased crop productivity resulted in increased frequency of extreme traits that eroded diversity for a number of plant attributes making the present day crop genotypes vulnerable to changes in environmental conditions, biotic and abiotic stresses. The early domesticates and wild relatives of crop plants are rich sources of diversity and exhibit better performance under harsh climatic conditions as well as under high pathogen loads. The plant breeders have realized the need of broadening the genetic base of cultivated genotypes and have made genuine efforts to explore alien-diversity to breed genotypes for challenging environmental conditions, improved yield and quality. It is evident from these efforts that incorporation of the alien chromatin into the cultivated background is an important tool to improve plant productivity. In this chapter, we deal with available sources of diversity, methods of alien introgression, available breeding material and its implications in characterization of the alien genes, successful examples of alien-introgression and their contribution to the crop improvement.

Keywords AB-QTL • Alien introgression • Backcross breeding • Domestication • Gene pool • Wild relatives

9.1 Introduction

Advent of agriculture in the Neolithic era set the foundation of human civilization and initially, the domestication process carved wild plants into their domesticated counterparts through extensive selection for morphological traits that directly or

N. Kumar • S. Rustgi, Ph.D. (✉)
Department of Crop and Soil Sciences, Washington State University,
271 Johnson Hall, Pullman, WA 99164, USA
e-mail: nkumar@wsu.edu; rustgi@wsu.edu

A. Pratap and J. Kumar (eds.), *Alien Gene Transfer in Crop Plants, Volume 1: Innovations, Methods and Risk Assessment*, DOI 10.1007/978-1-4614-8585-8_9,
© Springer Science+Business Media New York 2014

indirectly benefitted humans (Salamini et al. 2002). Polyploidy also played a major role in domestication process of some of the major crop plants, and aided by extensive artificial selection, it resulted in the fertility barriers between crop plants and their respective wild relatives (Zeven 1980; Feuillet et al. 2008; Matsuoka 2011). However, these activities gradually narrowed down the genetic base of different crop plants. The crop monocultures over vast areas in past led to some major crop failures and famines due to disease epidemics, and can lead to disasters even in future. For example, more than 80 % of the wheat cultivated around the globe at present is susceptible to *Ug99* and breakdown to this important disease could lead to a major disaster (Stokstad 2007). Similarly, changes in environments, such as an increase in temperature by a few degrees, will push the wheat belt up in the arctic region since most of the presently cultivated wheat is incapable of thriving in high temperatures, especially during anthesis period (Black 2006; Lobell et al. 2008; Swaminathan 2009). Monoculture of crop varieties has also lead to a plateau in terms of the grain yield, because the existing genotypes for most of the crops have already reached their maximum yield potential with improved agronomical practices (Conway 1997; Swaminathan 2010; Jacques and Jacques 2012). Therefore, there is a need to expand the genetic base of the crop plants using exotic germplasm and wild relatives (Fig. 9.1). This has been well realized by the breeders, who started exploring this option as early as in 1950s in the "cytogenetics era" and developed several important genetic resources using the germplasm and exotic material (Gill and Friebe 2009). As a result, several important characters including resistance to biotic and abiotic stresses, improvement in different components of grain yield and/or quality have been transferred to the cultivated plants over the years. These materials have been characterized and evaluated using the existing technologies, while the procedures have been improved tremendously to increase the level of precision in their analysis (Kole 2011a, b, c). The improvement in the technology has allowed identification of even submicroscopic segments of foreign DNA dubbed as "cryptic introgressions" (Kuraparthy et al. 2007, 2009). The ways to break the segments introgressed from the alien species have also improved, and in recent years, the technologies are also available which help in reducing the proportion of linkage drag and also isolation of the underlying gene(s) in specific cases (Gill et al. 2011; Cao et al. 2011). In this chapter we discuss about the methods and technologies of alien introgression in light of the agronomically important traits transferred into several crop plants.

9.2 Sources of Diversity Available for Introgression Breeding

Due to the limited genetic diversity in the elite cultivated gene pools of most of the crop plants, the major source of diversity available to the breeders comes from the wild relatives. Based on the degree of sexual compatibility, the source of diversity or "gene pool" used for the improvement of a given species was classified into the primary, secondary, and tertiary gene pools (Harlan and de Wet 1971) (see Fig. 9.2).

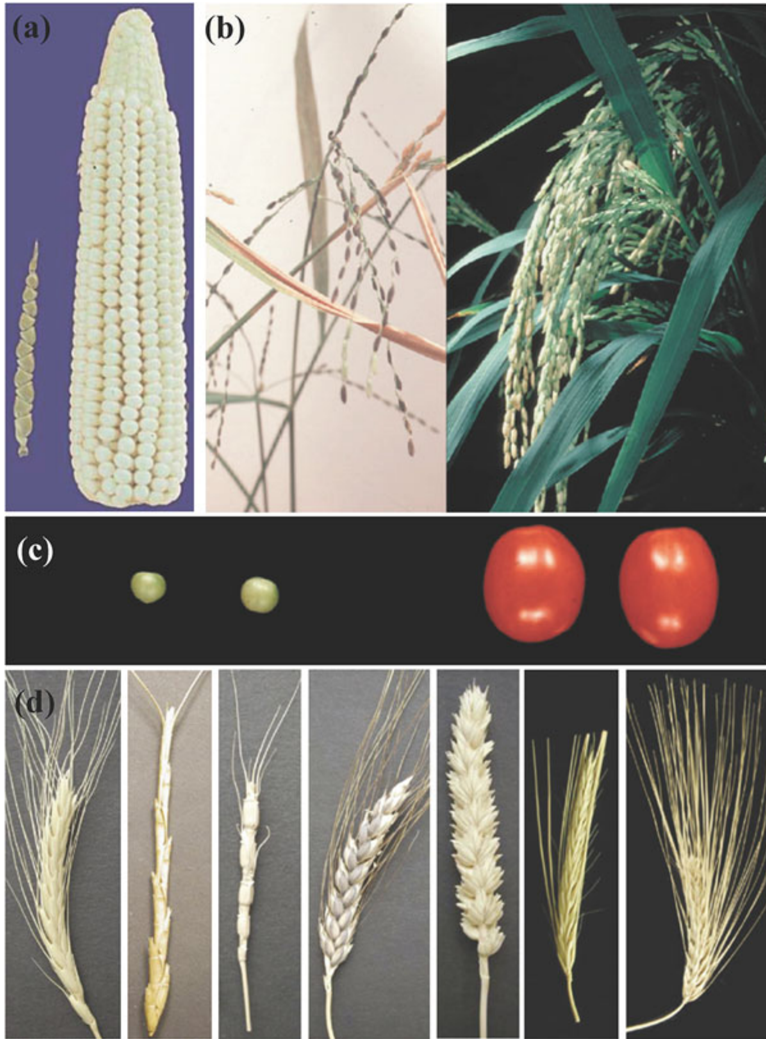

Fig. 9.1 A glimpse of genetic diversity existing in the exotic gene pools of maize, rice, tomato, wheat, and barley. (**a**) Mature inflorescence, or "ear" of teosinte (*Zea mays* ssp. *mexicana*) (*left*) and of modern corn (*Z. mays* ssp. *mays* L.) (*right*). (**b**) Mature panicle of wild rice species *Oryza rufipogon* (*left*) and of modern rice (*O. sativa*) cultivar from China (*right*). (**c**) Green fruits of the wild species *Lycopersicon pennellii* (*left*), and the lycopene-rich red fruits of *L. esculentum* (*right*). (**d**) The grain-bearing spikes of wild and domesticated forms of wheat and barley. From *left* to *right*: *Triticum urartu* (A^uA^u), *Aegilops speltoides* (SS), *Ae. tauschii* (DD), *T. turgidum* ssp. *dicoccoides* (AABB), *T. aestivum* (bread wheat, AABBDD), *Hordeum spontaneum* (HH), and six rowed *H.* vulgare (HH)

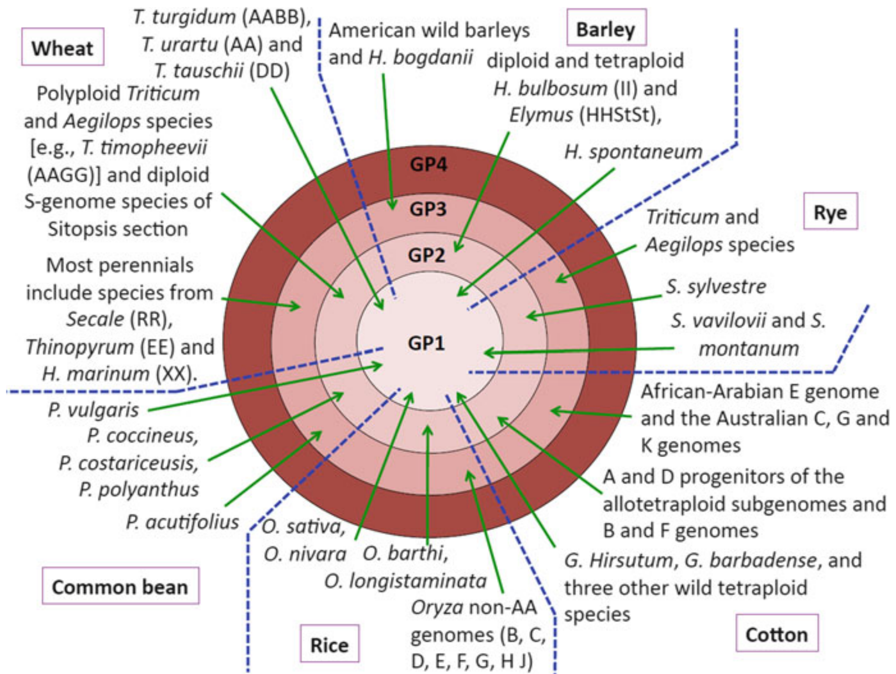

Fig. 9.2 Sources of genetic variation or "gene pools" for crop improvement. Harlan and de Wet (1971) proposed the crop gene pools to guide the germplasm use by the plant breeders, where it was suggested to first utilize the germplasm in the gene pool 1 (GP1) and proceed outwards. The number of species in each of the GPs that plant breeders can use varies among crops

Nevertheless, with the advent of genetic-engineering procedures there are essentially no barriers restricting the flow of genes across the genome boundaries, and thus, a fourth category including unrelated organisms known as quaternary gene pool was includes (Suslow et al. 2002). The first two categories mostly include intra- and/or interspecific (rarely intergeneric) introgressions, where in the latter case the two species must share at least one common genome to allow normal recombination to take place. The third category includes intergeneric introgressions that involve crossing between two different genera where normal recombination does not take place. These different categories are elaborated further using specific examples of cereals in the tribe Triticeae (Feuillet et al. 2008), rice (Lu and Snow 2005), cotton (Stewart 1995), soybean (Singh 2007), and *Brassica* (Branca and Cartea 2011).

9.2.1 Primary Gene Pool (GP-1)

This group comprises species that are interfertile and have no problems related to the fertility of the crossed progeny. Species in the primary gene pool include landraces, early domesticates and wild species that hybridize directly with the cultivated types.

In addition, species listed in this category have chromosomes homologous to the cultivated types, which allow homologous recombination to take place, thus these lines can be easily exploited in breeding and selection schemes. For example, in the case of the polyploid cotton the primary gene pool includes the wild, commensal and feral forms of *Gossypium hirsutum* and *G. barbadense* and three other wild tetraploid cotton species. Similarly, in case of soybean (2n = 40) the primary gene pool is only comprised of its wild annual progenitor *Glycine soja* (2n = 40), and for rapeseed it represents *Brassica carinata*, *B. juncea*, *B. napus* ssp. *napus*, *B. napus* ssp. *napobrassica*, and *B. napus* ssp. *pabularia*. In case of polyploid wheat, *Triticum turgidum* (AABB) and diploid donors of the A and D subgenomes [*T. urartu* (AA) and *T. tauschii* (DD)] represent the primary gene pool. In barley (HH) and rye (RR), the primary gene pools include very diverse but sexually compatible, diploid progenitors *Hordeum spontaneum* and *Secale vavilovii*, *S. montanum*, respectively.

9.2.2 Secondary Gene Pool (GP-2)

The members of this group include both cultivated and wild relatives of a crop species and are more distantly related and encounter crossability barriers. However, the hybrids in this group are just sufficiently fertile to allow gene flow. The secondary gene pool of cotton comprises of the extant relatives of the A and D subgenome progenitors as well as the B and F genomes. Similarly, the secondary gene pool of *Brassica* is comprised of *B. nigra*, *B. oleracea*, and *B. rapa*, and of wheat contains polyploid species that share at least one homologous genome with the cultivated types. Gene transfer from these species is possible through homologous recombination when the target gene is located on the homoeologous genome. This includes polyploid *Triticum* and *Aegilops* species, such as *T. timopheevii* (AAGG) and the diploid S-genome (related to the B genome) species from the *Sitopsis* section of *Aegilops*. For barley and rye, this includes diploid and tetraploid *H. bulbosum* (II) and *Elymus* (HHS^tS^t), and *S. silvestre*, respectively. However, *G. max* does not have a secondary gene pool.

9.2.3 Tertiary Gene Pool (GP-3)

Members of this group mostly face fertility problem when crossed with the members of GP-1. Since homologous recombination cannot be exploited, the gene transfers are really difficult, result in lethality, sterility, and other abnormalities, and require use of special strategies (such as embryo rescue, use of bridge species, irradiation, and gametocidal chromosomes) to overcome these difficulties (see below). Species in the tertiary gene pool contain more distantly related diploids and polyploids. They possess none of the cultivated species genome constitutions. This group in cotton includes the African-Arabian E genome and the Australian C, G, and K genomes, and in wheat includes most members of the Triticeae that are not

within the primary or secondary gene pools. A large proportion in this group comprises perennials and for wheat includes important species from *Secale* (RR), *Thinopyrum* (EE), and *H. marinum* (XX). For barley, the American wild barleys and *H. bogdanii* fall in this category, whereas for rye, the tertiary pool would include *Triticum* and *Aegilops* species. The tertiary gene pool in soybean includes 26 wild perennial species of the subgenus *Glycine*, and in rapeseed includes genera belonging to 36 cytodemes capable of genetic flux, such as *Diplotaxis, Enarthrocarpus*, *Eruca*, *Erucastrum*, *Hirschfeldia*, *Rhynchosinapis*, *Sinapis*, *Sinapodendron*, and *Trachystoma*.

9.2.4 Quaternary Gene Pool (GP-4)

The definition for the quaternary gene pool is quite controversial, according to some it incorporates incompatible related species, whereas according to others it incorporates any synthetic strain whose nucleic acid (DNA or RNA) frequency does not occur in nature. According to yet another definition, any organism from microbes to humans fall in this category where gene transfer is not possible by pollination or tissue culture and is only feasible through genetic transformation.

9.3 Tools and Techniques Used for Gene Introgression

Exploitation of secondary and tertiary gene pool requires application of specific cytogenetic procedures broadly described as “chromosome engineering” (c.f. Gupta and Tsuchiya 1991; Ceoloni and Jauhar 2006; Chan 2010). Traditionally the gene transfer takes place in the following steps: (1) addition of the whole alien genome (i.e., the production of amphiploids), (2) addition or substitution of a pair of alien chromosome (i.e., production of addition or substitutions lines), and (3) introgression of a segment of alien chromosome (i.e., production of recombinant chromosome line with the desired gene or the production of translocation lines). Though induction of homoeologous paring causes translocation, in several instances it occurred spontaneously (e.g., see Gill and Friebe 2002).

Introgressions from the alien species mostly take place in the form of Robertsonian translocations, which may occur naturally or can be induced through induction of pairing between homoeologous chromosomes (Mujeeb-Kazi and Rajaram 2002). For instance, mutations in *Pairing homoeologous 1* (*Ph1*) gene (*ph1b* in bread wheat and *ph1c* in durum wheat), responsible for diploid like pairing behavior of the polyploidy wheat, were extensively used to transfer genes from alien species and to reduce linkage drag in the introgressed alien segments (Mujeeb-Kazi and Rajaram 2002). The *PrBn* gene identified in *Brassica* is shown to have somewhat similar function, and mutations in this gene are expected to exhibit similar effects in *Brassica* (Jenczewski et al. 2003). Another homoeologues pairing suppressor *Ph2*

was also identified in bread wheat, but it has milder effect in comparison to the *Ph1* gene, and has rarely been exploited to induce homoeologous pairing in bread wheat (Ceoloni and Donini 1993). Different accessions of *Aegilops speltoides*, *Ae. longissima*, *Ae. peregrina*, and *Ae. kotschyi* are shown to induce homoeologous chromosome pairing by suppression of the *Ph1* gene activity and were used to some extent for transferring genes to the cultivated wheat (Kilian et al. 2011). The *Ph* suppressor genes *Su1-Ph1* (also known as Ph^I) and *Su2-Ph1* were, respectively, assigned to *Ae. speltoides* chromosomes 3S and 7S, and the *Su1-Ph1* was transferred from *Ae. speltoides* to the bread wheat genome (Dvorak et al. 2006). This Ph^I stock is an efficient inducer of homoeologous pairing since Ph^I gene is dominant and epistatic to the wheat *Ph1* gene (Chen et al. 1994). The other methods, which are more generally used to induce translocation and to transfer alien segment, are X-ray or gamma ionizing irradiation, which induces random chromatin translocation between chromosomes. Ability of gametocidal genes for breaking chromosomes was also used as a method for transferring alien segments in wheat, especially for production of wheat–rye translocations (Jauhar 2006).

Transfer of genes within the primary gene pool is affected by dominant nature of crossability inhibitor (*Kr*) genes in wheat. This markedly reduces the efficiency of successful production of interspecific hybrids. Four such genes (*Kr1* on 5BL, *Kr2* on 5AL, *Kr3* on 5D, and *Kr4* on 1A; *Kr1* having the largest effect on crossability) have been identified in most elite European wheat varieties, which have made the transfer of novel traits from exotic germplasm in to elite varieties difficult (Mujeeb-Kazi and Rajaram 2002). Therefore, in the presence of these crossability inhibitor genes, special cytogenetic procedures described above need to be applied to transfer genes within the primary gene pool.

9.4 Populations Developed Using Alien Sources and Their Applications

9.4.1 Advanced Backcross (AB) Population

Advanced backcross population was initially used by Tanksley and Nelson (1996) for mapping the genes/QTL introgressed from wild relatives. The advanced backcross populations (BC_2/BC_3) are developed from first crossing between improved cultivated lines and unadapted germplasm and subsequent back crossing with improved cultivated lines. AB populations comprising of different introgression lines carry the genetic variation in wild relatives (i.e., valuable QTLs/genes from unadapted germplasm) in the background of elite breeding lines. Several genes/QTL controlling agronomically important traits transferred from wild relatives have been dissected successfully in a number of crop plants including wheat, rice, tomato, barley, maize, and cotton (Table 9.1). The main purpose of using AB generations is to precisely measure effects of the individual QTLs by reducing size of the alien

Table 9.1 List of advanced-backcross QTL studies conducted in various crop plants

Salient features	Alien/other source	Reference
Tomato		
Horticultural traits	*L. pimpinellifolium*	Tanksley et al. (1996)
	L. peruvianum	Fulton et al. (1997)
	L. hirsutum	Bernacchi et al. (1998a)
	L. pimpinellifolium and *L. hirsutum*	Bernacchi et al. (1998b)
		Fulton et al. (2000)
	L. parviflorum	
Biochemical properties	–	Fulton et al. (2002)
Quality traits	Cherry tomato	Chaïb et al. (2006)
Ascorbic acid	*S. pennellii* and *S. habrochaites*	Stevens et al. (2007)
Rice		
Agronomic traits	*O. rufipogon*	Xiao et al. (1998)
	O. grandiglumis	Yoon et al. (2006), Xie et al. (2008)
Heading date	*O. sativa* Nipponbare	Yamamoto et al. (2000)
Yield and morphological traits	*O. rufipogon*	Thomson et al. (2003)
Grain quality traits	*O. rufipogon*	Septiningsih et al. (2003b)
Blast resistance	*O. rufipogon*	Wu et al. (2004)
	O. glaberrima	Liu et al. (2004)
	–	Manosalva et al. (2009)
Grain quality and grain morphology	*O. glaberrima*	Li et al. (2004)
Yield and yield contributing traits	*O. rufipogon*	Septiningsih et al. (2003a), Marri et al. (2005), Cheema et al. (2008)
Barley		
Yield and yield contributing traits	*H. vulgare* ssp. *spontaneum*	Pillen et al. (2003), Li et al. (2006)
Spot blotch resistance		Yun et al. (2006)
Morphological and agronomic traits	Harrington/OUH602	Gyenis et al. (2007)
Photoperiodic response	*H. vulgare* ssp. *spontaneum*	von Korff et al. (2004)
Powdery mildew, leaf rust, and scald resistance	ISR42-8	von Korff et al. (2005)
Agronomic traits		von Korff et al. (2006)
Malting parameters		von Korff et al. (2008)
Powdery mildew and leaf rust	*H. vulgare* ssp. *spontaneum* ISR42-8	Schmalenbach et al. (2008)
Agronomic traits		Schmalenbach et al. (2009)
Yield traits		Saal et al. (2011)
Yield and yield contributing traits	*H. spontaneum*	Eshghi et al. (2013)
Wheat		
Yield and yield contributing traits	Synthetic Wheat "W-7984"	Huang et al. (2003)

(continued)

Table 9.1 (continued)

Salient features	Alien/other source	Reference
Agronomic traits	Synthetic Wheat "XX86" Synthetic Wheat "TA 4152-4"	Huang et al. (2004), Narasimhamoorthy et al. (2006)
Quality traits	Synthetic Wheat "Syn022"	Kunert et al. (2007)
Leaf rust resistance	Synthetic Wheat "Syn022L"	Naz et al. (2008)
Maize		
Adventitious root formation under flooding conditions	Maize cv. Mi29 × teosinte *Z. nicaraguensis*	Mano et al. (2009)
Cotton		
Fiber length	*G. barbadense* and *G. hirsutum*	Chee et al. (2005a, b)
Fiber quality	*G. barbadense* and *G. hirsutum*	Draye et al. (2005)

chromatin by repeated backcrossing. This allows to avoid the confounding effect of deleterious wild alleles for domestication-related traits such as sterility, seed shattering, undesirable growth habit, and small fruits that could interfere with the measurements of yield and other field performance traits (Tanksley and Nelson 1996). Moreover, if epistasis interaction(s) are contributing to the effect, it is more likely to dissect the effect in the AB progeny than in the F_2 population, because the assembly of favorable epistatic alleles formed by the conventional breeding is expected to breakdown by the recombination events taking place during the backross cycles (Allard 1996). The AB-QTL analysis is best suited to annual inbred cultivars of crops rather than long-generation perennial crops, because the time required to develop an AB population and/or near-isogenic lines is prohibitively long in perennials and outbreeding crops. Following section describes a few examples of use of AB populations in QTL studies of selected crop plants (see Wang and Chee 2010; Grandillo and Tanksley 2005 for details).

9.4.1.1 Tomato

In this crop, genetic variability for fruit size and shape traits in the wild tomato species *Lycopersicon pimpinellifolium* "LA1589" was exploited through AB population-based QTL analysis (Tanksley et al. 1996). Several QTLs for horticultural traits were mapped, and a few NILs were developed. In another AB-QTL mapping study, performed for 35 traits, eight stable QTLs for fruit weight were identified in BC_2 and followed through BC_3 and BC_4 generations (Fulton et al. 1997). The results of QTL analysis showed that wild alleles contribute for horticulturally beneficial traits, albeit the wild (donor) parent has an allover inferior phenotype (Table 9.1). In a similar study, Bernacchi et al. (1998a) used AB-QTL mapping approach to transfer

and map 19 horticultural traits from wild species *L. hirsutum* "LA1777" using BC_2 and BC_3 populations (Table 9.1). The results showed that many QTLs transferred from *L. hirsutum* contribute for the superior alleles increasing total yield, soluble solid, and other traits. However, the overall phenotype of the donor parent is inferior. Later in another study, Bernacchi et al. (1998b) developed NILs having introgressions of wild chromatin carrying alleles originating from two donor species *L. hirsutum* (LA1777) and *L. pimpinellifolium* (LA1589). Evaluation of the agronomic performance of the NILs showed that the ranges of per-location gains over the elite control were from 6 to 59 % for different traits. Using BC_3 populations derived from an interspecific cross between an elite tomato inbred and the wild species *L. parviflorum* detected 199 QTLs for 30 horticultural traits, and at least one QTL each identified for 19 traits carried *L. parviflorum* allele with agronomically favorable effect (Fulton et al. 2000).

The use of AB-QTL approach in tomato is not limited to the horticultural traits but was also used to transfer and dissect important quality traits such as flavor. To study genetics of this complex trait, four AB populations were developed and used to identify QTLs responsible for the biochemical properties such as sugar and organic acid content, which contribute to the flavor of tomatoes (Fulton et al. 2002). Later this approach was used to identify QTLs for ascorbic acid content using three different populations developed using tomato related wild species or subspecies (Stevens et al. 2007). Majority of the QTLs detected during these studies were stably expressed in multiple environments.

9.4.1.2 Rice

Rice is represented by two cultivated species viz., *Oryza sativa* L. and *O. glaberrima* Steud and 21 wild species, and the *O. sativa* L. is further divided into two subspecies *indica* and *japonica* (Oka 1958; Khush 1997; Veasey et al. 2004). In rice, AB-QTL approach has been extensively used to improve agronomical performance of the cultivated rice, because genetic bottlenecks imposed during domestication and through modern breeding practices significantly reduced genetic variability for most of the agronomical traits in the modern rice cultivars (Kovach and McCouch 2008). Thus, a wide array of alien species was used as source of genetic diversity to improve a number of agronomically important traits in large number of studies (see Brar and Khush 1997; Wang and Chee 2010). For example, an accession of *O. rufipogon* was used to develop an interspecific BC_2 testcross population. Though the used accession of this species was phenotypically inferior to cultivated rice for 12 targeted traits, its backcross progeny revealed transgressive segregants better than elite parent for all studied traits (Xiao et al. 1998). The AB populations (BC_2) were also developed using *O. rufipogon*, *O. glaberrima*, and *O. grandiglumis* to transfer and dissect the yield and yield component traits (Xiao et al. 1996; Thomson et al. 2003; Septiningsih et al. 2003a,b; Li et al. 2004; Marri et al. 2005), heading date (Yamamoto et al. 2000), and blast resistance (Wu et al. 2004; Yoon et al. 2006) (Table 9.1).

9.4.1.3 Barley

The genus *Hordeum* comprises 32 species and 45 taxa including diploid, tetraploid, and hexaploid species (von Bothmer et al. 1995). Wild barley, *H. vulgare* ssp. *spontaneneum,* is the progenitor of cultivated barley and is fully interfertile with the cultivated species. AB populations have been developed and used to introgress favorable alleles from wild to the cultivated barley (see Wang and Chee 2010). Albeit most of the wild species are phenotypically inferior in nature, have been successfully used to improve agronomical traits such as yield and yield components, malting quality and resistance to diseases such as leaf rust, powdery mildew, and scald resistance (Baum et al. 2003; Pillen et al. 2003, 2004; Grando et al. 2005; von Korff et al. 2005, 2006, 2008; Gyenis et al. 2007; Wang and Chee 2010; Eshghi et al. 2013).

9.4.1.4 Wheat

Bread or common wheat (*Triticum aestivum* L.) is an allohexaploid, which represents 95 % of the cultivated wheat, and the remaining 5 % of it is represented by the allotetraploid durum wheat (*T. turgidum* ssp. *durum*) (Dubcovsky and Dvorak 2007). AB populations in wheat have been used to exploit diversity existing in the exotic gene pool for traits such as yield and yield components, quality and disease resistance (Table 9.1). The identification of QTLs for yield and its component traits using a backcross population was accomplished in crosses made between synthetic wheat lines (W7984 and XX86) and German wheat cultivars (Huang et al. 2003, 2004).

9.4.1.5 Maize

AB-QTL analysis was performed in maize to identify QTLs for the traits of agronomical importance by using a population derived from a cross between two inbred lines, namely RD6502 and RD3013 (Ho et al. 2002). As many as, four QTLs were detected for grain yield, six for grain moisture content, and three for plant height, where favorable allele for each QTL was contributed by RD3013. In the same study, the four QTLs identified for grain yield were selected as target introgressions to develop BC_3 testcross families. Later, a few other AB-QTL studies were performed in maize for yield contributing and developmental traits, and utility of the wild relatives in improving performance of the cultivated maize was demonstrated (Wang and Chee 2010).

9.4.1.6 Cotton

The genus *Gossypium* consists of 45 diploid ($2n=2x=26$) and five tetraploid ($2n=4x=52$) species out of which only four species (*G. herbaceum, G. arboreum, G. hirsutum*, and *G. barbadense*) are under cultivation (Fryxell 1992; Wendel and Cronn 2003). Among these *G. hirsutum* and *G. barbadense* are the two most widely

cultivated tetraploid species. For using the superior fiber quality of *G. barbadense* and high fiber yield of *G. hirsutum,* BC_3F_2 population derived from an intercross between *G. hirsutum* cv. Tamcot 2111 and *G. barbadense* cv. Pima F_6 was used to dissect the QTLs (Chee et al. 2005a, b). These studies led to the detection of 28 QTLs for fiber length. The QTLs were distributed on 15 different chromosomes and explained 8–28 % of the phenotypic variation for the fiber length, while *G. barbadense* was shown to have lower fiber length in comparison with the *G. hirsutum*, *G. barbadense* alleles at 64 % of the detected QTLs contributed for the increased fiber elongation (see Table 9.1).

9.4.2 Development and Use of Introgression Line (IL) or Chromosome Segment Substitution Line (CSSL) Libraries

To facilitate breeding of environmentally stable highly productive elite cultivars several alien introgression and/or substitution lines have been developed in a number of crop plants. These introgression and/or substitution lines served as mapping populations for gene localization as well as germplasm to breed for the superior traits. These lines have been successfully developed in a variety of crop plants and used to decipher genetic basis of several agronomical traits (Brar and Khush 1997; Singh and Hymowitz 1999; Gill et al. 2006; Lippman et al. 2007). Elucidation of the molecular mechanism underlying many of these traits allowed creation of new gene combinations to improve plant performance (Ashikari and Matsuoka 2006; Lippman et al. 2007; Sacco et al. 2013).

In the last decade, a number of concepts were proposed to breed for the elite genotypes using alien introgression lines. According to the concept of Galinat (1999), reverting back to the state (of more genetic diversity) from where one has started selecting for the traits of interest (resulting in the present state of reduced genetic diversity) is defined as "reverse breeding," and is one of the ways of creating unique gene combinations. McCouch (2004) defined it as "smart breeding" as it recycles "old genes" to produce new combinations with better output in terms of phenotypic gains. The objective of "reverse breeding" was to combine various plant attributes by taking some traits from the exotic germplasm and the others from the elite cultivars. These objectives can be achieved by the development of introgression line (IL) libraries, where a single homozygous segment from wild species is introgressed into a uniform, cultivated background with the help of molecular markers (Zamir 2001). The ILs thus produced assist in fine mapping as well as pyramiding of the desired characters in a single genotype by crossing different ILs (Fernie et al. 2006). Some success has already achieved in this direction, for instance, in tomato Gur and Zamir (2004) created an IL library by crossing *Solanum pennellii* (wild tomato) with *S. lycopersicum* var. M82 (cultivated tomato) followed by selecting for lines each carrying a unique introgressed segment from the wild tomato in the uniform recipient parent background. Later, they used these ILs to "pyramid" three independent yield contributing genomic regions derived from the wild species

into a single elite genotype. Their results demonstrated that an approach based on biodiversity, which takes advantage of genetic variability existing in the wild relatives of tomato can be used to create genotypes that can outperform any commercially available hybrid variety both in terms of yield and drought resistance. Recently, to improve fruit quality traits in tomato, Schauer and coworkers screened an IL library, where segments determining variation in quantities of different metabolites from *S. pennellii* were introgressed in the genetic background of *S. lycopersicum* var. Roma. This effort not only allowed mapping of 1,200 new “metabolite QTLs” (mQTLs) but also revealed a sizable correlation between the fruit quality traits and other yield-related traits (Schauer et al. 2006). The localization of fruit quality and yield contributing traits to specific genomic regions will assist in development of an ideal genotype using “breeding by design” and its implementation by crossing desirable ILs following MAS (Giovannoni 2006). Similar approach can be applied in rice where two IL populations carrying donor segments from the wild species (*O. glumaepatula* and *O. meridionalis*) in the genetic background of cultivated rice (*O. sativa*), cv. Taichung 65 were developed (Yoshimura et al. 2010). More recently, 105 ILs were developed in cotton by crossing a wild cotton species, *G. darwinii* Watt with four upland cotton cultivars. Eventhough these ILs are in four different genetic backgrounds, high similarity coefficients existed between lines derived from different parents. When these ILs and their upland cotton parents were used for association mapping of fiber quality traits SSR markers were identified to be linked with five fiber quality traits. Some of these associations were detected in multiple environments (Wang et al. 2012).

The CSSLs were also developed in the orphan crops like peanut. A marker-assisted backcrossing strategy was used to produce a population of 122 CSSLs, derived from a cross between wild synthetic allotetraploid (*Arachis ipaënsis* × *A. duranensis*)^{4x} and the cultivated peanut (*A. hypogaea* L.) var. Fleur11. The 122 CSSLs showed good coverage of the peanut genome with target wild chromosome segments averaging 39.2 cM in length. To demonstrate the utility of these lines, four traits were evaluated in a subset of 80 CSSLs. The marker trait association analysis allowed assignment of 42 QTLs including 14 QTLs for plant growth habit, 15 QTLs for height of the main stem, and 12 QTLs for plant spread and one for flower color (Fonceka et al. 2012).

9.4.3 Development and Use of Recombinant Inbred Chromosomal Lines (RICLs), Recombinant Inbred Chromosome Substitution Lines (RICSLs), and Recombinant Chromosome Substitution Lines (RCSLs)

Typically the genome of chromosome substitution lines is comprised of all chromosomes derived from a recurrent parent except for a single chromosome originating from the donor parent. To determine the position of genes on the substitution chromosome and to reduce confounding effect of the epistatic interactions

(background noise), RICL populations were developed for individual substitution chromosomes by crossing a substitution line with the recurrent parent (Law 1966; Joppa et al. 1997). These RICL populations were successfully used to clone genes contributing for a number of agronomically important traits (Frary 2000; Yano 2001; Song et al. 1995; Uauy et al. 2006). RICLs are common in wheat where they have had a long history (Sears 1953). However, due to the laborious process of developing these lines only a few QTL mapping studies have so far been conducted using RICLs. In general RICLs allow more efficient dissection of complex traits than any other commonly used biparental mapping population (Campbell et al. 2003; Distelfeld et al. 2006; Rustgi et al. 2013). For instance, using an RICL carrying an introgression from *T. dicoccoides* (wild emmer wheat), Uauy et al. (2006) identified and cloned a gene (*Gpc-B1*) responsible for senescence, grain protein, zinc, and iron content in bread wheat.

In barley two different RICL populations were developed, one from a cross between a wild barley accession H602 (*H. vulgare* ssp. *spontaneum*) and a malting barley cv. Haruna Nijo (*H. vulgare* ssp. *vulgare*) (Hori et al. 2005), and the other from the cross of an accession of *H. spontaneum* (Caeserea 26-24, from Israel) with a North American malting barley cv. Harrington (*H. vulgare* subsp. *vulgare*) (Matus et al. 2003). The former population consists of 144 RICLs (BC_3 generation), which was scored for five qualitative and nine quantitative traits and genotyped with 85 DNA markers and a total of 18 QTLs for nine qualitative traits. In spite of general inferior agronomic performance of wild barley, several H602 QTL alleles contributed for the agronomically positive effects (Hori et al. 2005). The later populations of 140 RICLs obtained using two backcrosses with the recurrent parent and six generations of self-pollination (BC_2F_6) was used to find markers associated with a variety of traits. For instance, Matus et al. (2003) evaluated 140 RICLs for yield contributing traits, malting quality traits, and domestication traits. Significant differences among the RICLs for all measured phenotypes were detected. The phenotypic effects of the introgressions were assessed using association analysis. In this study *H. vulgare* ssp. *spontaneum* was observed to be contributing for the favorable alleles for agronomic and malting quality traits. In another study, a subset of 80 RICLs was evaluated for grain yield and plant height in six different environments. The association analysis performed using the phenotype data and 47 SSR markers identified 21 chromosomal regions that showed high correlations with differences in grain yield, plant height, and/or yield adaptability. In about one-fourth of the cases, the *H. spontaneum* (donor) contributed for the favorable alleles (Inostroza et al. 2009).

Another study evaluated a subset of 80 RICLs for grain yield and plant height in six different environments. The marker-trait association analysis showed that *H. spontaneum* (donor) contributed for the favorable alleles (Inostroza et al. 2009). Using this set of 80 RICLs, accumulation of fructans was observed to be higher in RICLs in comparison with the recurrent parent Harrington. This provided evidence that the introgressions from the wild ancestor (*H. vulgare* ssp. *spontaneum*) into cv. Harrington are responsible for increasing the terminal drought tolerance in cultivated barley (Méndez et al. 2011; del Pozo et al. 2012).

9.4.4 Naturally Introgressed Lines (Admixed Populations)

Not only the experimentally created populations but also the natural admixed populations in crop plants and horticultural species can be used to map genes and to serve as germplasm to breed for agronomically important traits. Naturally occurring hybrids of wheat and *Aegilops*, wheat and rye, maize and *Teosinte*, *Sorghum bicolor* and *S. halepense*, *japonica* and *indica* rice could be utilized to study traits of adaptive significance (such as weediness, biotic and abiotic stresses), domestication-related traits, consequences of gene flow between wild plants and their cultivated relatives and its influence on evolutionary process (Hegde and Waines 2004; Arrigo et al. 2011).

These natural populations exhibit linkage disequilibrium (LD) induced by admixture of subpopulations with different ancestries. Efforts have therefore been made to develop tools to utilize these natural admixed populations to study marker-trait associations. In this connection, the concept of mapping by admixture linkage disequilibrium (MALD) came into existence, and has been widely discussed since the late 1980s (Reich and Patterson 2005). Feasibility of admixture mapping in plants was studied using natural admixed populations of sunflower (Rieseberg and Buerkle 2002; Rieseberg et al. 1999), poplar (Lexer et al. 2007), and cocoa (Marcano et al. 2007). Two of these studies demonstrated successful implementation of admixture mapping in tree species, where desired crosses are often difficult to obtain.

In sunflower (*Helianthus*), introgression regions, i.e., chromosomal blocks from wild species contributed to reproductive isolation and affected the pollen sterility when an admixture population comprising 139 individuals from three zones of wild sunflower hybrid (*H. annuus* × *H. petiolaris*) were analyzed (Rieseberg et al. 1999).

In poplar (*Populus* spp.), Lexer et al. (2007) suggested that admixture LD among highly informative SSR markers in *Populus* hybrid zones should permit precise estimation of marker ancestry in hybrids leading to the identification of individual chromosome blocks with high adaptive and/or selective values (fitness effects) in the admixed populations, and hybrid populations in other European river valleys could serve as independent “replicates”. Admixture should also allow the detection of associations among markers and quantitative phenotypic traits or ecological habitat factors. This information would be of great interest not only for evolutionary biology but also for applied breeding programs.

Similarly, in cocoa an admixed population derived from the mixture of “Criollo” and “Trinitario” (originally a hybrid of “Criollo” and “Forastero”) was used for admixture mapping. Two different collections, one collected 25 years ago represented by 150 individuals and the other collected from the contemporary stands of cocoa represented by 1,000 individuals was used for admixture mapping. The former allowed estimation of ancestral allelic states and the latter was used for a genome-wide scan of marker trait associations, after screening individuals for the presence of “Criollo/Trinitario” alleles at 10 SSR loci. This information has allowed minimization of the above set of 1,000 individuals selected on the basis of morphological traits to a set of 291 individuals. A genome-wide scan of 291 individuals

using 101 SSR markers allowed identification of 15 genomic regions involved in seed and fruit weight variations. These loci corresponded to ten previously identified QTLs and five novel ones (Marcano et al. 2007). The results suggested potential implication of admixture mapping in the dissection of complex traits, and identification of markers-tags for component loci that can be used in future breeding applications.

9.4.5 Nested Association Mapping (NAM) Population

To assist mapping of genes/QTLs contributing for agronomically relevant traits, Edward Buckler and his coworkers developed a single unified mapping population daubed as nested-association mapping (NAM) population. The NAM population allows mapping of genes by both linkage- and LD-based approaches. The first NAM population was developed in maize by crossing one common parent B73 with 25 diverse maize founder inbreds followed by selfing the F_1s to generate 25 F_2 populations. These F_2 populations were advanced through single seed descent (SSD) to generate 25 half-sib RIL populations each with 200 RILs, collectively constituting an NAM population of 5,000 RILs. This population takes in account both historic and recent recombination events, thus will allow simultaneous deception of marker trait association and validation of identified markers (Yu et al. 2008).

Later the similar NAM design was applied in barley to explore the genetic diversity for flowering time (FT) in the wild barley. For this purpose a barley NAM population consisting of 1,500 BC_1S_3 lines was obtained by crossing barley cultivar Barke with 25 highly divergent wild barley accessions. For genotypic characterization, the NAM population is currently being genotyped with 9,000 Infinium SNPs and selected candidate genes with known functions in the FT control. The data obtained will be used to carry out an association genetics screen to localize new wild barley QTLs, which are associated with the expression of FT. The new exotic QTL alleles will help to broaden the genetic diversity of the elite barley gene pool. Additionally, the exotic QTL alleles will shed further light on the genetic network of FT control in cereals (Klaus Pillen personal communication).

9.5 Role of Exotic Germplasm in Improving Performance of the Elite Counterparts

9.5.1 Breeding for Abiotic Stress Resistance

Changes in the climatic conditions such as rising temperature and uncertain precipitation pattern in combination with the deteriorating edaphic conditions are one of the major causes of yield stagnation (Gupta et al. 2012; Cossani and Reynolds 2012; Lobell and Gourdji 2012). Keeping in view the increasing global population it is

important to develop crops that are less demanding and can perform stably in suboptimal growth conditions. It has been realized well before that the wild relatives of the cultivated plants perform more stably under stress conditions, and have since been extensively exploited to breed for abiotic stress resistance (Trethowan and Mujeeb-Kazi 2008). One of the specific examples includes the 1BL.1RS translocation (Rajaram et al. 1983; Villareal et al. 1995). This translocation of the long arm of chromosome 1B (1BL) with the short arm of rye (*Secale cereale* L.) chromosome 1R (1RS) was first discovered in the winter wheat cv. Kavkaz, and was later transferred to spring bread wheat cv. Veery (Rajaram et al. 1990). It was observed that the lines carrying this translocation exhibit vigorous root growth, and maintain high grain yield in drought prone conditions and on Zn-deficient or acidic soils (Schlegel et al. 1997; Ribeiro-Carvalho et al. 1997; Manske and Vlek 2002; Ehdaie et al. 2003). The yield advantage of 1RS translocation lines was attributed to the increase in root biomass that increases uptake of water and nutrients from the soil (Ehdaie et al. 2003; Snape et al. 2007; Ehdaie and Waines 2008). In general the 1BL.1RS carrying lines have inferior industrial quality, although later it became possible to break the association between agronomically inferior attributes from the superior characters (Lukaszewski 2000). Homoeologous recombinants of 1RS with 1BS were used for precise mapping the genes/QTLs for root traits in wheat (Sharma et al. 2009, 2011).

Similarly, tolerance to salinity has been transferred from the wild Triticeae species to the cultivated wheat (Zan-Min 2003). For instance, *Thinopyrum bessarabicum* was crossed to bread wheat and the salt tolerance of the resulting amphiploid was found to be significantly improved relative to the recurrent parent (King et al. 1997). The translocated segment of another species (*Th. junceum*) of same genera into the cultivated wheat background significantly improved the salt tolerance (Wang et al. 2003). The salt-tolerant grasses *Lophopyrum elongatum* (Host) Love and *Elytrigia pontica* (Podp.) Holub also offer potential for improving the salt tolerance of wheat (Dvorak and Knott 1974; Dvorak et al. 1988). The wild relatives such as *Ae. geniculata* found in harsh environments are reported to have low carbon isotope discrimination and therefore high water-use efficiency (Zaharieva et al. 2001), and can be potential source of variability for drought and heat tolerance.

In a recent study, physiological and molecular consequences of an alien chromosome segment (7DL) introgression from a wild wheat relative, *Agropyron elongatum* into cultivated wheat (*T. aestivum*) background were investigated. The wheat translocation lines showed significantly improved water stress adaptation, and higher root and shoot biomass compared to the control genotypes. Enhanced uptake of water and nutrients from the soil due to higher root biomass enabled the translocation line to maintain more favorable gas exchange and carbon assimilation levels relative to the wild type during water stress. Transcriptome analysis allowed identification of candidate genes associated with root development. Two of these candidate genes were mapped to the site of translocation on chromosome 7DL. Based on the candidate gene analysis, brassinosteroid signaling pathway was predicted to be involved in the novel root responses observed in the *Agropyron* translocation line (Placido et al. 2013).

9.5.2 Breeding for Biotic Stress Resistance

Application of modern genomic tools in combination with the cytogenetic procedures has enabled the plant breeders to identify and transfer desirable disease resistance genes from exotic to the cultivated gene pool of different crops. In wheat significant progress has been achieved in identifying and transferring alien genes conferring resistance against a number of diseases such as leaf, stem and stripe rusts, powdery mildew, bunts, and smuts (Knott 1989; Pienaar 1990; Jiang and Gill 1994; Friebe et al. 1996; Bommineni and Jauhar 1997; Gill et al. 2006, 2011). Almost all genes conferring resistance against various diseases are of alien origin, in particular the ones providing resistance against *Fusarium* head blight (FHB), stem rust (*Sr*), powdery mildew (*Pm*), and leaf rust (*Lr*) (c.f. Brown-Guidera et al. 1996; Fedak et al. 1997; Singh et al. 2008; Chen et al. 2013; Timonova et al. 2013). As this topic is widely reviewed in several recent articles in the following paragraph we focus on a few major examples (see Table 9.2).

Thirty accessions of *T. araraticum* were identified to show resistance against seven different diseases and two of them showed resistance against both FHB and leaf rust (Fedak 1999). In *T. monococcum,* resistance for eyespot disease was found (Murray et al. 1994), which was later transferred to the cultivated gene pool (Cadle et al. 1997; Sheng et al. 2012). Thus, in order to make use of the insect/pest resistance identified in several different alien sources a large collection of synthetic wheat lines were developed at the International Maize and Wheat Improvement Center, CIMMYT, and are currently being used in several breeding programs around the globe (Mujeeb-Kazi and Rajaram 2002). Use of alien gene in rice and maize for insect/pest and disease resistance have been used to develop the introgressed lines (Amante-Bordeos et al. 1992; Brar and Khush 1997; Rahman et al. 2009) (presented in Sect. 4.1.2, and Tables 9.1 and 9.2).

In rice alien genes for insect/pest resistance have been introgressed in cultivated varieties using AB populations (Amante-Bordeos et al. 1992; Brar and Khush 1997; Rahman et al. 2009). Similarly, in maize introgression lines were developed for southern leaf blight, northern leaf blight, and grey leaf spot resistance using marker-assisted backcrossing (Tables 9.1 and 9.2).

In *Brassica*, a large number of *B. oleracea* accessions were screened for resistance against verticillium wilt, and a good source of resistance was identified for this devastating disease (Happstadius et al. 2003). Similarly, other wild *Brassica* species in particular *B. vollosa* and *B. incana* were also used as the sources of resistance against insect-pests (Ellis et al. 2000). Similarly, in cotton due to low genetic variability only tertiary gene pool has been exploited (by bridge crossing) to improve disease resistance (Brubaker and Brown 2003; McFadden et al. 2004).

9.5.3 Breeding for Yield and Yield Contributing Traits

In wheat, the key examples of alien introgressions that contributed to yield improvement include (1) introgressions from *Ae. umbellulata*, which saved the US wheat crop

Table 9.2 Wild sources used to improve desirable traits in crop plants

Crop	Trait under consideration	Donor	Recipient	Reference
Biotic and abiotic stress				
Tomato	Yellow leaf curl resistance	*S. chilense*	–	de Castro et al. (2013)
Brassica	Resistance to cabbage aphid *Brevicoryne brassicae*	*B. incana* and *B. villosa*	–	Ellis et al. (2000)
	Resistance to verticellium wilt	*B. incana*	–	Happstadius et al. (2003)
	Powdery mildew resistance	*B. carinata*	*B. oleracea*	Tonguc and Griffiths (2004)
	Characterization of mustard aphid *Lipaphis erysimi* resistance gene	*B. fruticulosa*	*B. juncea*	Kumar S et al. (2011)
	Aphid resistance	*B. fruticulosa*	*B. rapa*	Atri et al. (2012)
	Blackleg resistant	*B. rapa* ssp. *Sylvestris*	*B. napus*	Yu et al. (2012)
Wheat	Elimination of large amount of goatgrass chromatin surrounding stem rust resistance gene *Sr39*	*Ae. speltoides*	*T. aestivum*	Niu et al. (2011)
	Powdery mildew resistance	*Dasypyrum villosum* syn. *Haynaldia villosa*	*T. aestivum*	Cao et al. (2011)
	Powdery mildew resistance	*H. villosa*	*T. aestivum*	Chen et al. (2013)
	Leaf rust resistance	*T. timopheevii*	*T. aestivum*	Timonova et al. (2013)
	Stripe rust resistance	*Psathyrostachys huashanica* Keng	*T. aestivum*	Du et al. (2013)
	Improved water stress adaptation, higher root and shoot biomass	*Ag. elongatum*	*T. aestivum*	Placido et al. (2013)
Rice	Brown plant hopper resistance	*O. minuta*	*O. sativa*	Rahman et al. (2009)
	Bacterial blight and brown plant hopper resistance	*O. minuta*	*O. sativa*	Guo et al. (2013)
Maize	Southern and northern leaf blight and grey leaf spot resistance	Elite Maize line	Cultivated maize	Belcher et al. (2012)
Cotton	*Fusarium* wild resistance	*G. sturtianum* *G. australe*	*G. hirsutum*	McFadden et al. (2004)

(continued)

Table 9.2 (continued)

Crop	Trait under consideration	Donor	Recipient	Reference
Yield and yield contributing traits				
Rice	Yield contributing traits	*O. rufipogon*	*O. sativa*	Tian et al. (2006, 2007)
	Increased grain yield	*O. minuta*	*O. sativa*	Linh et al. (2008)
	Yield and yield contributing traits	*O. rufipogon*	*O. sativa*	Fu et al. (2010)
	Morphological and yield contributing traits	*O. minuta*	*O. sativa*	Guo et al. (2013)
Wheat	Leaf rust resistance *Lr19* and high grain yield	*Ag. elongatum*	*T. aestivum*	Reynolds et al. (2001)
	Increased grain yield	*T. dicoccoides*	*T. aestivum*	Simmonds et al. (2008)
	Increased number of florets and kernels	*Ag. cristatum*	*T. aestivum*	Wu et al. (2006)
	Yield and yield contributing traits	*Th. bessarabicum*	*T. aestivum* cv. Chinese Spring	Qi et al. (2010)
Quality traits				
Maize	Kernel protein content and amino acid composition	*Z. mays* ssp. *mexicana*	*Z. mays* cv. Ye515	Wang et al. (2008a)
Wheat	Soft grain texture	*H. villosa*	*T. aestivum*	Zhang et al. (2012)
Rice	High head rice percentages and grain amylose content	*O. rufipogon*	*O. sativa* cv. MR219	Fasahat et al. (2012)
Brassica	High glucoraphanin content	*B. villosa*	-	Sarikamis et al. (2006)
Cotton	Fiber quality traits	*G. darwinii* Watt	Cultivated cotton	Wang et al. (2012)
Other interesting traits				
Brassica	Yellow seed trait	*Sinapis alba*	*B. napus*	Li et al. (2012)
Wheat	Polycarpic life history	*Th. elongatum*	*T. aestivum* cv. Chinese Spring	Lammer et al. (2004)
	Blue wheat grain color	*Th. bessarabicum*	*T. aestivum*	Shen et al. (2013)

from catastrophic failure in 1960s (Sears 1956, 1972), and (2) the 1B/1R introgression from rye that conferred resistance against a number of insect-pests, tolerance to the acid soils, and contributed for increased plant biomass and grain yield. After its discovery in the 1990s, the 1B/1R translocation was transferred to the majority of

world wheat varieties and is still present in the leading wheat varieties under cultivation throughout the globe (Ammar et al. 2004). Similarly, another translocation from *T. dicoccoides* is present in many of the leading Europe wheat varieties, for example, "Robigus." A few other examples of yield improvement in wheat using alien introgression(s) include the 7Ag.7DL introgression from *Ag. elongatum*, which contributed for both increase in grain yield and biomass in different wheat backgrounds (Reynolds et al. 2001). This introgression can be tracked with the help of molecular markers associated with the leaf rust resistance gene *Lr19*. Non-glaucousness introduced from *T. dicoccoides* delayed plant senescence and prolong grain-filling duration in wheat leading to an increase in grain yield (Simmonds et al. 2008). Development of synthetic wheats at CIMMYT also showed great potential of alien species in breeding for improved grain yield (Trethowan and Mujeeb-Kazi 2008).

The AB-QTL approach was successfully used in rice to introgress alien segments contributing for improved grain yield into the cultivated rice background (Xiao et al. 1998). In this study, chromosome segments introgressed from a Malaysian accession (IRGC 105491) of *O. rufipogon* increased the grain yield of cultivated rice in the BC_3 testcross progeny. Two other studies used the same *O. rufipogon* accession to cross with different recipient genotypes, which also resulted in increased grain yield in backcross progenies (Thomson et al. 2003; Septiningsih et al. 2003a,b).

9.5.4 Breeding for Nutritional Quality

Grain protein content is one of the major determinants of the nutritional quality of agricultural crops. It has a direct influence on the quality of end products and also on the health of consumers. In cereal grains, GPC ranges from 10 to 12 % of dry wt. and in legume seeds it is 20–40 % (Shewry and Halford 2002). Wild emmer wheat, *T. turgidum* ssp. *dicoccoides*, accession FA-15-3 was identified as a source for high GPC (Avivi 1978). Two decades after its discovery, Joppa et al. (1997) developed alien chromosome substitution lines using FA-15-3, which not only allowed transfer of the gene(s) responsible for high GPC from exotic to the elite background but also assisted in assignment of the effect to wheat chromosome 6B. Later a map-based cloning approach was followed to clone the gene (*Gpc-B1*) responsible for high GPC which was found to encode an NAC transcription factor designated as "No Apical Meristem1" (NAM1) protein (Uauy et al. 2006). It was also reported that the tetraploid wheat *T. turgidum* ssp. *dicoccoides* has a functional *Gpc-B1* allele, while the modern tetraploid and hexaploid wheat cultivars have a deletion at this locus or a nonfunctional copy (Uauy et al. 2006). This is one of the success stories of nutritional enhancement in wheat where the gene conferring high GPC was originally identified in a wild species, successfully transferred to the cultivated tetraploid and hexaploid wheats, and with the availability of functional markers for the gene *Gpc-B1*, has been successfully used in developing wheat genotypes with enhanced GPC without any yield penalty (Kumar J et al. 2011; Balyan et al. 2013).

In maize, introgression lines were developed from a cross between *Zea mays* ssp. *mexicana*, a close wild relative of cultivated maize, and an elite maize inbred line Ye515 for increasing protein content and nutritional value. Kernel protein contents in the progeny ranged from 7.89 to 12.44 % with considerable variability in the proportions of different seed storage proteins and amino acid composition. Protein content and amino acid composition (specifically Lys content) of some introgression lines was significantly higher and improved than that of Ye515. The results demonstrated that these introgressions had great potential in improving the protein content and composition of maize grains (Wang et al. 2008a, b, 2012).

In rice, a limited backcrossing procedure was utilized to introgress genes associated with grain quality traits from *O. rufipogon* (IRGC 105491), a wild rice relative, to the cultivated rice *O. sativa* cv. MR219. Advanced breeding lines carrying *O. rufipogon* introgressions showed significantly higher head rice (amount of whole grain rice) percentages (70–88 %), and two progenies showed higher amylose contents than MR219 (Fasahat et al. 2012).

9.5.5 Bio-Fortification of Mineral Nutrients Using Alien Species

Dietary deficiency of micronutrients, known as hidden hunger, affect more than 40 % of the world's population, especially in the developing nations (Welch and Graham 2002). Worldwide, more than 3 billion people living on staple crops suffer from micronutrient deficiencies (Welch and Graham 2004; Liu et al. 2006). Out of these, 2 billion people suffer from iron and zinc deficiencies alone. About one-third of humans in all age groups and populations, especially women and children, are severely affected by deficiency of key micronutrients, e.g., iron (Fe), zinc (Zn), iodine (I), selenium (Se), and vitamin A (Ghandilyan et al. 2006). Breeding for micronutrient enhancement has recently been seen as one of the major breeding objectives in many crop plants. Modern day cultivars of all major crops have limited variability for mineral content (Graham et al. 2001; Bouis 2003). However, wild germplasm of crops has been found to harbor sufficient variability for improvement of mineral content (Cakmak et al. 2000; Chavez et al. 2005; Vreugdenhil et al. 2005; Rawat et al. 2009a, b; White and Broadley 2009). Breeding for biofortification in wheat has been discussed in some recent reviews (Rawat et al. 2013; Balyan et al. 2013). In wheat, six tetraploid species including *Ae. kotschyi*, *Ae. peregrina*, *Ae. geniculata*, *Ae. ventricosa*, and *Ae. cylindrica* and a diploid species, *Ae. longissima* exhibited high grain Fe and Zn content that can be exploited for biofortification of Fe and Zn content in the elite bread and durum wheat cultivars (Rawat et al. 2009a). Few accessions of *Ae. kotschyi* were also used to make wide crosses with bread wheat to produce chromosome substitution lines (Rawat et al. 2011). The substitution lines carrying chromosomes 2S and 7S from *Ae. kotschyi* in common wheat background showed threefold increase in grain Fe and Zn contents

(Rawat et al. 2009b; Tiwari et al. 2010). Similarly, introgression of *Ae. peregrina* (a tetraploid with U and S genomes) segments in common wheat genome resulted in two- to threefold increase in the grain Fe and Zn content (Neelam et al. 2011). Disomic addition lines derived using *Ae. longissima*, *Ae. searsii*, *Ae. umbellulata*, *Ae. caudata*, *Ae. peregrina*, and *Ae. geniculata* showed increased grain Fe or Zn concentration of between 50 and 248 % compared with the recipient cultivar, Chinese Spring. Most of alien chromosomes addition lines with significantly higher grain Fe and/or Zn concentrations belonged to the U and S genotypes and homoeologous groups 1 and 2 chromosomes (Wang et al. 2011).

Selenium is an essential micronutrient for mammals (but not plants), being present as seleno cysteine in a number of enzymes. However, it is also toxic when present in excess (above ~600 μg d^{-1}). Cereals are major dietary sources of Se, where Se exists in high-selenium gluten with as much as 7 ppm. About 40–45 % of the Se is bound in as seleno methionine and/or seleno cysteine, and incorporation of seleno amino acids into plant proteins happens by a replacement of cysteine and methionine, often with a deleterious consequence on the plant's health (Hawkesford and Zhao 2007).

In a recent study no significant genotypic variation in grain Se density among cultivated bread or durum wheat, triticale, or barley varieties was observed. However, a wild wheat relative, *Ae. tauschii* and rye showed higher grain Se concentration (42 and 35 %, respectively) than the other evaluated lines (Lyons et al. 2005).

9.6 Conclusions and Future Prospects

The need to look for the source of biodiversity outside the cultivated or elite gene pools has been realized since long and resulted in establishment of germplasm banks around the globe. For instance there are almost 500,000 wheat accessions available worldwide in gene banks, encompassing landraces, non-domesticated species, and advanced and obsolete cultivars (Ortiz et al. 2008). Similarly, more than 80,000 rice germplasm accessions are available in national and international collections (Chang 1984; Jackson 1997). In several other crops also a large number of germplasm accessions and wild genetic resources are available with the gene banks. This vast reservoir of biodiversity must be explored and utilized to address the issue of feeding the global population in changing environmental conditions. Advanced genetic and genomic approaches are creating new opportunities in large-scale omics (genomics, metabolomics, proteomics, transcriptomics, and ionomics), with great potentials to enable a thorough understanding of genome structure and behavior. The new genotyping platforms based on array-based (Gupta et al. 2013) and next-generation sequencing (NGS) methods (Seifollah et al. 2013) will make genome-wide molecular marker discovery and genotyping faster and cost effective, which will consequently improve the use of wild germplasm to breed crops for future needs. It is expected that genomic selection (GS) enabled by genotyping-by-sequencing (GBS) will allow precise estimation of

recombination events (Poland and Rife 2012), which will significantly improve the precision with which alien introgressions could be tracked or located. These new resources will accelerate the isolation of genes underlying key agronomic traits and provide a new generation of gene-specific diagnostic markers for breeding and genes for genetic manipulations (GM). These advances in marker technology will also enable the plant breeders to engineer genotypes with the desired attributes following the concept of "Breeding by Design" (Peleman and van der Voort 2003). Although efficiently relating gene sequence variation with functional variation remains a challenge, a better knowledge of genome structure, recombination distribution and regulation will accelerate the development of strategies such as "allele replacement" through sequence-specific homologous recombination to reducing linkage drag. With the improvements in the transformation protocols in different crops, it is now possible to transfer cis-/transgenes in the desired genetic backgrounds. Further, with the advent of zink-finger (ZF) and transcription activator like effector (TALE) nucleases now it is feasible to target gene(s) to desired genomic locations (Baker 2012). With these emerging technologies we can foresee a whole new era in crop improvement which will circumvent the present day limitations of using elite gene pools and will allow to breed for genotypes with desired attributes.

Acknowledgements This work was supported by the Life Sciences Discovery Fund (LSDF) Grant 3143956, and Washington Grain Commission (WGC) Grants to NK (3019-3450-3019-5449 and 3019-7452) and SR (13C-3019-3590). The authors would also like to thank Mrs. Richa Gemini for assisting with the references.

References

Allard RW (1996) Genetic basis of the evolution of adaptedness in plant. In: Tigerstedt A (ed) Adaptation in plant breeding. Kluwer, The Netherlands, pp 1–6

Amante-Bordeos A, Sitch LA, Nelson R, Dalmacio RD, Oliva NP, Aswidinnoor H, Leung H (1992) Transfer of bacterial blight and blast resistance from the tetraploid wild rice *Oryza minuta* to cultivated rice, *Oryza sativa*. Theor Appl Genet 84:345–354

Ammar K, Mergoum M, Rajoram S (2004) The history and evolution of triticale. In: Megoum M, Gomez-Macpherson H (eds) Triticale improvement and production. Rome, Italy, pp 1–11, FAO Plant Production and Protection Series No. 179

Arrigo N, Guadagnuolo R, Lappe S, Pasche S, Parisod C, Felber F (2011) Gene flow between wheat and wild relatives: empirical evidence from *Aegilops geniculata Ae. neglecta* and *Ae. triuncialis*. Evol Appl 4:685–695

Ashikari M, Matsuoka M (2006) Identification, isolation and pyramiding of quantitative trait loci for rice breeding. Trends Plant Sci 11:344–350

Atri C, Kumar B, Kumar H, Kumar S, Sharma S, Banga SS (2012) Development and characterization of *Brassica juncea*–fruticulosa introgression lines exhibiting resistance to mustard aphid (*Lipaphis erysimi* Kalt). BMC Genet 13:104

Avivi L (1978) High protein content in wild tetraploid *Triticum dicoccoides* Korn. In: Ramanujam S (ed) Proceedings of the 5th international wheat genetics symposium. Indian Society of Genetics and Plant Breeding, New Delhi, India, pp 372–380

Baker M (2012) Gene-editing nucleases. Nat Methods 9:23–26

Balyan HS, Gupta PK, Kumar S, Dhariwal R, Jaiswal V, Tyagi S, Agarwal P, Gahlaut V, Kumari S (2013) Genetic improvement of grain protein content and other health-related constituents of wheat grain. Plant Breed. doi:10.1111/pbr.12047

Baum M, Grando S, Backes G, Jahoor A, Sabbagh A, Ceccarelli S (2003) QTLs for agronomic traits in the Mediterranean environment identified in recombinant inbred lines of the cross "Arta"×*H. spontaneum* 41-1. Theor Appl Genet 107:1215–1225

Belcher AR, Zwonitzer JC, Santa Cruz J, Krakowsky MD, Chung CL, Nelson R, Arellano C, Balint-Kurti PJ (2012) Analysis of quantitative disease resistance to southern leaf blight and of multiple disease resistance in maize, using near-isogenic lines. Theor Appl Genet 124:433–445

Bernacchi D, Beck-Bunn T, Emmatty D, Eshed Y, Inai S, Lopez J, Petiard V, Sayama H, Uhlig J, Zamir D, Tanksley S (1998a) Advanced backcross QTL analysis of tomato. II. Evaluation of near-isogenic lines carrying single-donor introgressions for desirable wild QTL-alleles from L. hirsutum and L. pimpinellifolium. Theor Appl Genet 97:170–180

Bernacchi D, Beck-Bunn T, Eshed Y, Lopez J, Petiard V, Uhlig J, Zamir D, Tanksley S (1998b) Advanced backcross QTL analysis in tomato. I. Identification of QTLs for traits of agronomic importance from Lycopersicon hirsutum. Theor Appl Genet 97:381–397

Black R (2006) New crops needed to avoid famines. Environment correspondent, BBC News website, http://news.bbc.co.uk/2/hi/science/nature/6200114.stm.

Bommineni VR, Jauhar PP (1997) Wide hybridization and genome relationships in cereals: an assessment of molecular approaches. Maydica 42:81–105

Bouis HE (2003) Micronutrient fortification of plants through plant breeding: can it improve nutrition in man at low cost? Proc Nutr Soc 62:403–411

Branca F, Cartea E (2011) Brassica. In: Kole C (ed) Wild crop relatives: genomic and breeding resources, Vol. Oilseeds. Springer-Heidelberg, Dordrecht, pp 17–36

Brar DS, Khush GS (1997) Alien introgression in rice. Plant Mol Biol 35:35–47

Brown-Guidera GL, Cox TS, Gill BS, Hatchett JH, Bockus WW, Leath S, Peterson CJ, Thomas JB, Zever P (1996) Evaluation of a collection of wild *timopheevi* wheat for resistance to disease and anthropod pests. Plant Dis 80:928–933

Brubaker CL, Brown AHD (2003) The use of multiple alien chromosome addition aneuploids facilitates genetic linkage mapping of the *Gossypium* G genome. Genome 46:774–791

Cadle MM, Murray TD, Jones SS (1997) Identification of resistance to *Pseudocercosporella herpotrichoides* in *Triticum monococcum*. Plant Dis 81:1181–1186

Cakmak I, Ozkan H, Braun HJ, Welch RM, Romheld V (2000) Zinc and iron concentrations in seeds of wild, primitive, and modern wheats. Food Nutr Bull 21:401–403

Campbell BT, Baenziger PS, Gill KS, Eskridge KM, Budak H, Erayman M, Dweikat I, Yen Y (2003) Identification of QTL and environmental interaction associated with agronomic traits on chromosomes 3A of wheat. Crop Sci 43:1493–1505

Cao A et al (2011) Serine/threonine kinase gene *Stpk-V*, a key member of powdery mildew resistance gene *Pm21*, confers powdery mildew resistance in wheat. Proc Natl Acad Sci U S A 108:7727–7732

Ceoloni C, Donini P (1993) Combining mutations for the two homoeologous pairing suppressor genes *Ph1* and *Ph2* in common wheat and in hybrids with alien Triticeae. Genome 36:377–386

Ceoloni C, Jauhar PP (2006) Chromosome engineering of the durum wheat genome: Strategies and applications of potential breeding value. In: Singh RJ, Jauhar PP (eds), Genetic Resources, Chromosome Engineering, and Crop Improvement, vol 2: Cereals, CRC Press-Taylor & Francis Group, Boca Raton, pp 27–59

Chaïb J, Lecomte L, Buret M, Causse M (2006) Stability over genetic backgrounds, generations and years of quantitative trait locus (QTLs) for organoleptic quality in tomato. Theor Appl Genet 112:934–944

Chan SWL (2010) Chromosome engineering: power tools for plant genetics. Trends Biotechnol 28:605–610

Chang TT (1984) Conservation of rice genetic resources: luxury or necessity? Science 224: 251–256

Chavez AL, Sanchez T, Jaramillo G, Bedoya JM, Echeverry J, Bolanos EA, Ceballos H, Iglesias CA (2005) Variation of quality traits in cassava roots evaluated in landraces and improved clones. Euphytica 143:125–133

Chee P, Draye X, Jiang CX, Decanini L, Delmonte TA, Bredhauer R, Smith CW, Paterson AH (2005a) Molecular dissection of interspecific variation between *Gossypium hirsutum* and *Gossypium barbadense* (cotton) by a backcross-self approach: I. Fiber elongation. Theor Appl Genet 111:757–763

Chee P, Draye X, Jiang CX, Decanini L, Delmonte TA, Bredhauer R, Smith CW, Paterson AH (2005b) Molecular dissection of phenotypic variation between *Gossypium hirsutum and Gossypium barbadense* (cotton) by a backcross-self approach: III. Fiber length. Theor Appl Genet 111:772–781

Cheema KK, Navtej SB, Mangat GS, Das A, Vikal Y, Brar DS, Khush GS, Singh K (2008) Development of high yielding IR64 × *Oryza rufipogon* (Griff.) introgression lines and identification of introgressed alien chromosome segments using SSR markers. Euphytica 160: 401–409

Chen P, Chunfang Y, Yin H, Shengwei C, Bo Z, Aizhong C, Xiue W (2013) Radiation-induced translocations with reduced *Haynaldia villosa* chromatin at the *Pm21* locus for powdery mildew resistance in wheat. Mol Breed 31:477–484

Chen PD, Tsujimoto H, Gill BS (1994) Transfer of *Ph^{I}* genes promoting homoeologous pairing from *Triticum speltoides* to common wheat. Theor Appl Genet 88:97–101

Conway G (1997) The doubly green revolution: food for all in the 21st century. Cornell University Press—Technology & Engineering, USA, p 344

Cossani CM, Reynolds MP (2012) Physiological traits for improving heat tolerance in wheat. Plant Physiol 160:1710–1718

de Castro AP, Julian O, Diez MJ (2013) Genetic control and mapping of *Solanum chilense* LA1932, LA1960 and LA1971-derived resistance to tomato yellow leaf curl disease. Euphytica 190:203–214

del Pozo A, Castillo D, Inostroza L, Matus I, Méndez AM, Morcuende R (2012) Physiological and yield responses of recombinant chromosome substitution lines of barley to terminal drought in a Mediterranean-type environment. Annl Appl Biol 160:157–167

Distelfeld A, Uauy C, Fahima T, Dubcovsky J (2006) Physical map of the wheat high-grain protein content gene *Gpc-B1* and development of a high-throughput molecular marker. New Phytol 169:753–763

Draye X, Chee P, Jiang CX, Decanini L, Delmonte TA, Bredhauer R, Smith CW, Paterson AH (2005) Molecular dissection of inter specific variation between *Gossypium hirsutum* and *G. barbadense* (cotton) by a backcross-self approach: II. Fiber fineness. Theor Appl Genet 111:764–771

Du W, Wang J, Lu M, Sun S, Chen X, Zhao J, Yang Q, Jun Wu J (2013) Molecular cytogenetic identification of a wheat–*Psathyrostachys huashanica* Keng 5Ns disomic addition line with stripe rust resistance. Mol Breed. doi:10.1007/s11032-013-9841-0

Dubcovsky J, Dvorak J (2007) Genome plasticity a key factor in the success of polyploid wheat under domestication. Science 316:1862–1865

Dvorak J, Deal KR, Luo MC (2006) Genome integrity and transmission discovery and mapping of wheat *Ph1* suppressors. Genetics 174:17–27

Dvorak J, Edge M, Ross K (1988) On the evolution of the adaptation of *Lophopyrum elongatum* to growth in saline environments. Proc Natl Acad Sci U S A 85:3805–3809

Dvorak J, Knott DR (1974) Disomic and ditelosomic additions of diploid *Agropyron elongatum* chromosomes to *Triticum aestivum*. Can J Genet Cytol 16:399–417

Ehdaie B, Waines JG (2008) Larger root system increases water—nitrogen uptake and grain yield in bread wheat. In: Appels et al. (eds) 11th international wheat genetics symposium. Sydney University Press, Brisbane, QLD, Australia, pp 659.

Ehdaie B, Whitkus RW, Waines JG (2003) Root biomass, water-use efficiency, and performance of wheat-rye translocations of chromosomes 1 and 2 in spring bread wheat "Pavon". Crop Sci 43:710–717

Ellis PR, Kiff NB, Pink DAC, Jukes PL, Lynn J, Tatchell GM (2000) Variation in resistance to the cabbage aphid (*Brevicoryne brassicae*) between and within wild and cultivated brassica species. Genet Resour Crop Evol 47:395–401

Eshghi R, Salayeva S, Ebrahimpour F, Rahimi M, Baraty M, Ojaghi J (2013) Advanced-backcross QTL analysis in hulless barley: I. Detection of exotic alleles for yield and yield components introgressed from Hordeum vulgare ssp. Spontaneum. Intl J Agri Crop Sci 5:95–100

Fasahat P, Muhammad K, Abdullah A, Wickneswari R (2012) Identification of introgressed alien chromosome segments associated with grain quality in *Oryza rufipogon* × MR219 advanced breeding lines using SSR markers. Genet Mol Res 11:3534–3546

Fedak G, Armstrong KC, Sinha RC, Gilbert J, Procunier JD, Miller JD, Pandeya R (1997) Wide crosses to improve *Fusarium* head blight resistance in wheat. Cereal Res Commun 25: 651–654

Fedak G (1999) Molecular aids for integration of alien chromatin through wide crosses. Genome 42:584–591

Fernie AR, Tadmor Y, Zamir D (2006) Natural genetic variation for improving crop quality. Curr Opin Plant Biol 9:196–202

Feuillet C, Langridge P, Waugh R (2008) Cereal breeding takes a walk on the wild side. Trends Genet 24:24–32

Fonceka D, Tossim HA, Rivallan R, Vignes H, Lacut E et al (2012) Construction of chromosome segment substitution lines in peanut (*Arachis hypogaea* L.) using a wild synthetic and QTL mapping for plant morphology. PLoS One 7:e48642

Frary A (2000) *fw2.2*: a quantitative trait locus key to the evolution of tomato fruit size. Science 289:85–88

Friebe B, Jiang J, Raupp WJ, McIntosh RA, Gill BS (1996) Characterization of wheat alien translocations conferring resistance to diseases and pests: current status. Euphytica 91:59–87

Fryxell PA (1992) A revised taxonomic interpretation of *Gossypium* L., (Malvacea). Rheedea 2:108–165

Fu Q, Zhang P, Tan L, Zhu Z, Ma D, Fu Y, Zhan X, Cai H, Sun C (2010) Analysis of QTLs for yield-related traits in Yuanjiang common wild rice (*Oryza rufipogon* Griff.). J Genet Genomics 37:147–157

Fulton TM, Beck-Bunn T, Emmatty D, Eshed Y, Lopez J, Petiard V, Uhlig J, Zamir D, Tanksley SD (1997) QTL analysis of an advanced backcross of *Lycopersicon peruvianum* to the cultivated tomato and comparisons with QTLs found in other wild species. Theor Appl Genet 95:881–894

Fulton TM, Bucheli P, Voirol E, López J, Péetiard V, Tanksley SD (2002) Quantitative trait loci (QTL) affecting sugars, organic acids and other biochemical properties possibly contributing to flavor, identified in four advanced backcross populations of tomato. Euphytica 127:163–177

Fulton TM, Grandillo S, Beck-Bunn T, Fridman E, Frampton A, Lopez J, Petiard V, Uhlig J, Zamir D, Tanksley SD (2000) Advanced backcross QTL analysis of a *Lycopersicon esculentum* × *Lycopersicon parviflorum* cross. Theor Appl Genet 100:1025–1042

Galinat WC (1999) Reverse maize breeding for high density populations. Maize Genet Newslet 73:91

Ghandilyan A, Vreugdenhil D, Aarts MGM (2006) Progress in the genetic understanding ofplant iron and zinc nutrition. Physiol Plant 126:407–417

Gill BS, Friebe B (2009) Cytogenetic analysis of wheat and rye genomes. In: Feuillet C, Muehlbauer GJ (eds) Genetics and Genomics of the Triticeae vol. 7. Springer, New York, pp 121–135

Gill BS, Friebe B, Raupp WJ, Wilson DL, Cox TS, Sears RG, Brown-Guedira GL, Fritz AK (2006) Wheat genetics resource center: the first 25 years. Adv Agron 89:73–135

Gill BS, Friebe BR, White FF (2011) Alien introgressions represent a rich source of genes for crop improvement. Proc Natl Acad Sci U S A 108:7657–7658

Gill BS, Friebe B (2002) Cytogenetics, phylogeny and evolution of cultivated wheats. In: Rajaram S, Curtis BC, Gomez Macpherson H (eds) Bread wheat—improvement and production. FAO, Rome, pp 71–88, Plant Production and Protection series No. 30

Giovannoni JJ (2006) Breeding new life into plant metabolism. Nat Biotechnol 24:418–419
Graham RD, Welch RM, Bouis HE (2001) Addressing micronutrient malnutrition through enhancing the nutritional quality of staple foods: principles, perspectives and knowledge gaps. Advance Agron 70:77–142
Grandillo S, Tanksley SD (2005) Advanced backcross QTL analysis: results and perspectives. In: Tuberosa R, Phillips RL, Gale M (eds) Proceedings of the international congress on the wake of the double helix: from the green revolution to the gene revolution, 27–31 May 2003. Bologna, Italy, pp 115–132, ©2005 Avenue media.
Grando S, Baum M, Ceccarelli S, Goodchild A, El-Haramein FL, Jahoor A, Backes G (2005) QTL for straw quality characteristics identified in recombinant inbred lines of a *Hordeum vulgare*×*H. spontaneum* cross in a Mediterranean environment. Theor Appl Genet 110:688–695
Guo SB, Wei Y, Li XQ, Liu KQ, Huang FK, Chen CH, Gao GQ (2013) Development and identification of introgression lines from the cross of *Oryza sativa* and *Oryza minuta*. Rice Sci. doi:10.1016/S1672-6308(13)60111-0
Gupta PK, Balyan HS, Gahlaut V, Kulwal PL (2012) Phenotyping, genetic dissection, and breeding for drought and heat tolerance in common wheat: status and prospects. Plant Breed Rev 36:85–168
Gupta PK, Rustgi S, Mir RR (2013) Array-based high-throughput DNA markers and genotyping platforms for cereal genetics and genomics. In: Gupta PK, Varshney RK (eds) Cereal genomics-II. Springer, New York
Gupta PK, Tsuchiya T (1991) Chromosome engineering in plants: genetics, breeding, evolution (developments in plant genetics and breeding). Elsevier Science, USA
Gur A, Zamir D (2004) Unused genetic variation can lift yield barriers in plant breeding. PLoS Biol 2: e245
Gyenis L, Yun SJ, Smith KP, Steffenson BJ, Bossolini E, Sanguineti MC, Muehlbauer GJ (2007) Genetic architecture of quantitative trait loci associated with morphological and agronomic trait differences in a wild by cultivated barley cross. Genome 50:714–723
Happstadius I, Ljungberg A, Kristiansson B, Dixelius C (2003) Identification of *Brassica oleracea* germplasm with improved resistance to *Verticillium* wilt. Plant Breed 122:30–34
Harlan JR, de Wet JMJ (1971) Towards a rational classification of cultivated plants. Taxon 20:509–517
Hawkesford MJ, Zhao FJ (2007) Strategies for increasing the selenium content of wheat. J Cer Sci 46:282–292
Hegde SG, Waines JG (2004) Hybridization and introgression between bread wheat and wild and weedy relatives in North America. Crop Sci 44:1145–1155
Ho JC, McCouch SR, Smith ME (2002) Improvement of hybrid yield by advanced backcross QTL analysis in elite maize. Theor Appl Genet 105:440–448
Hori K, Sato K, Nankaku N, Takeda K (2005) QTL analysis in recombinant chromosome substitution lines and doubled haploid lines derived from a cross between *Hordeum vulgare* ssp *vulgare* and *Hordeum vulgare* ssp *spontaneum*. Mol Breed 16:295–311
Huang XQ, Cöster H, Ganal MW, Röder MS (2003) Advanced backcross QTL analysis for the identification of quantitative trait loci alleles from wild relatives of wheat (*Triticum aestivum* L). Theor Appl Genet 106:1379–1389
Huang XQ, Kempf H, Ganal MW, Röder MS (2004) Advanced backcross QTL analysis in progenies derived from a cross between a German elite winter wheat variety and a synthetic wheat (*Triticum aestivum* L). Theor Appl Genet 109:933–943
Inostroza L, del Pozo A, Matus I, Castillo D, Hayes P, Machado S, Corey A (2009) Association mapping of plant height, yield, and yield stability in recombinant chromosome substitution lines (RCSLs) using *Hordeum vulgare* subsp. *spontaneum* as a source of donor alleles in a *Hordeum vulgare* subsp. *vulgare* background. Mol Breed 23:365–376
Jackson MT (1997) Conservation of rice genetic resources: the role of International RiceGenebank at IRRI. Plant Mol Biol 35:61–67
Jacques PJ, Jacques JR (2012) Monocropping cultures into ruin: the loss of food varieties and cultural diversity. Sustainability 4:2970–2997

Jauhar PP (2006) Cytogenetic architecture of cereal crops and their manupulation to fit human needs: opportunities and challenges. In: Singh RJ, Jauhar PP (eds) Genetic resources, chromosome engineering, and crop improvement: cereals, vol 2. CRC Press, Boca Raton, FL, pp 1–25

Jenczewski E, Eber F, Grimaud A, Huet S, Lucas MO, Monod H, Chevre AM (2003) *PrBn*, a major gene controlling homeologous pairing in oilseed rape (*Brassica napus*) haploids. Genetics 164:645–653

Jiang J, Gill BS (1994) Recent advances in alien gene transfer in wheat. Euphytica 73:199–212

Joppa LR, Changheng D, Hart GE, Hareland GA (1997) Mapping gene(s) for grain protein in tetraploid wheat (*Triticum turgidium* L.) using a population of recombinant inbred chromosome lines. Crop Sci 37:1586–1589

Khush GS (1997) Origin, dispersal, cultivation and variation of rice. Plant Mol Biol 35:25–34

Kilian B, Mammen K, Millet E, Sharma R, Graner A, Salamini F, Hammer K, Özkan H (2011) Aegilops. In: Kole C (ed) Wild crop relatives: genomic and breeding resources. Cereals. Springer, New York, pp 1–76

King IP, Forster BP, Law CN, Kant KA, Orford SE, Gorham J, Reader S, Miller TE (1997) Introgression of salt tolerance genes from *Thinopyrum bessarabicum* into wheat. New Phytol 137:75–81

Knott DR (1989) The wheat rusts—breeding for resistance. Theoretical and applied genetics monograph no. 12, Springer, Berlin.

Kole C (2011a) Wild crop relatives: genomic and breeding resources: legume crops and forages. Springer, New York

Kole C (2011b) Wild crop relatives: genomic and breeding resources: oilseeds. Springer, New York

Kole C (2011c) Wild crop relatives: genomic and breeding resources: cereals. Springer, New York

Kovach MJ, McCouch SR (2008) Leveraging natural diversity: back through the bottleneck. Curr Opin Plant Biol 11:193–200

Kumar S, Atri C, Sangha MK, Banga SS (2011) Screening of wild crucifers for resistance to mustard aphid, Lipaphis erysimi (Kaltenbach) and attempt at introgression of resistance gene(s) from *Brassica fruticulosa to Brassica juncea*. Euphytica 179:461–470

Kumar J, Jaiswal V, Kumar A, Kumar N, Mir RR, Kumar S, Dhariwal R, Tyagi S, Khandelwal M, Prabhu KV, Prasad R, Balyan HS, Gupta PK (2011). Introgression of a major gene for high grain protein content in some Indian bread wheat cultivars. Field Crop Research 123:226–233

Kunert A, Naz AA, Dedeck O, Pillen K, Léon J (2007) AB-QTL analysis in winter wheat: I. Synthetic hexaploid wheat (*T. turgidum* ssp. *dicoccoides* × *T. tauschii*) as a source of favourable alleles for milling and baking quality traits. Theor Appl Genet 115:683–695

Kuraparthy V, Chhuneja P, Dhaliwal HS, Kaur S, Gill BS (2007) Characterization and mapping of cryptic alien introgressions from *Aegilops geniculata* with new leaf rust and stripe rust resistance genes *Lr57* and *Yr40* in wheat. Theor Appl Genet 114:1379–1389

Kuraparthy V, Sood S, Gill BS (2009) Molecular genetic description of the cryptic wheat–*Aegilops geniculata* introgression carrying rust resistance genes *Lr57* and *Yr40* using wheat ESTs and synteny with rice. Genome 52:1025–1036

Lammer D, Cai X, Arterburn M, Chatelain J, Murray T, Jones S (2004) A single chromosome addition from *Thinopyrum elongatum* confers a polycarpic perennial habit to annual wheat. J Exp Bot 55:1715–1720

Law CN (1966) The location of genetic factors affecting a quantitative character in wheat. Genetics 53:487–498

Lexer C, Buerkle CA, Joseph JA, Heinze B, Fay MF (2007) Admixture in Europe *Populus* hybrid zones makes feasible the mapping of loci that contribute to reproductive isolation and trait differences. Heredity 98:74–84

Li A, Jiang J, Zhang Y, Snowdon RJ, Liang G, Wang Y (2012) Molecular and cytological characterization of introgression lines in yellow seed derived from somatic hybrids between *Brassica napus* and *Sinapis alba*. Mol Breed 29:209–219

Li J, Xiao J, Grandillo S, Jiang L, Wan Y, Deng Q, Yuan L, McCouch SR (2004) QTL detection for rice grain quality traits using an Interspecific backcross population derived from cultivated Asian (*O. sativa* L.) and African (*O. glaberrima* S.) rice. Genome 47:697–704

Li JZ, Huang XQ, Heinrichs F, Ganal MW, Röder MS (2006) Analysis of QTLs for yield components, agronomic traits, and disease resistance in an advanced backcross population of spring barley. Genome 49:454–466

Linh HL, Hang NT, Jin FX, Kang KH, Lee YT, Kwon K, Ahn SN (2008) Introgression of a quantitative trait locus for Spikelets per panicle from *Oryza minuta* to the *O. sativa* cultivar Hwaseongbyeo. Plant Breed 127:262–267

Lippman ZB, Semel Y, Zamir D (2007) An integrated view of quantitative trait variation using tomato interspecific introgression lines. Curr Opin Genet Dev 17:545–552

Liu B, Zhang S, Zhu X, Yang Q, Wu S, Mei M, Mauleon R, Leach J, Mew T, Leung H (2004) Candidate defense genes as predictors of quantitative blast resistance in rice. Mol Plant Microbe Interact 17:1146–1152

Liu ZH, Wang HY, Wang XE, Zhang GP, Chen PD, Liu DJ (2006) Genotypic and spike positional difference in grain phytase activity, phytate, inorganic phosphorus, iron, and zinc contents in wheat (*Triticum aestivum* L.). J Cereal Sci 44:212–219

Lobell DB, Burke MB, Tebaldi C, Mastrandrea MD, Falcon WP, Naylor RL (2008) Prioritizing climate change adaptation needs for food security in 2030. Science 319:607–610

Lobell DB, Gourdji SM (2012) The influence of climate change on global crop productivity. Plant Physiol 160:1686–1697

Lu B-R, Show AA (2005) Gene flow from genetically modified rice and its environmental consequences. BioScience 55:669–678

Lukaszewski AJ (2000) Manipulation of the 1RS.1BL translocation in wheat by induced homoeologous recombination. Crop Sci 40:216–225

Lyons G, Ortiz-Monasterio I, Stangoulis J, Graham R (2005) Selenium concentration in wheat grain: is there sufficient genotypic variation to use in breeding? Plant Soil 269:369–380

Mano Y, Omori F, Loaisiga CH, Bird RMK (2009) QTL mapping of above-ground adventitious roots during flooding in maize × teosinte "*Zea nicaraguensis*" backcross population. Plant Root 3:3–9

Manosalva PM, Davidson RM, Liu B, Zhu X, Hulbert SH, Leung H, Leach JE (2009) A germin-like protein gene family functions as a complex quantitative trait locus conferring broad-spectrum disease resistance in rice. Plant Physiol 149:286–296

Manske GGB, Vlek PLG (2002) Root architecture—wheat as a model plant. In: Waisel Y, Eshel A, Kafkafi U (eds) Plant roots: the hidden half. Marcel Dekker Inc, New York, pp 249–259

Marcano M, Pugh T, Cros E, Morales S, Portillo Páez EA, Courtois B, Glaszmann JC, Engels JM, Phillips W, Astorga C, Risterucci AM, Fouet O, González V, Rosenberg K, Vallat I, Dagert M, Lanaud C (2007) Adding value to cocoa (*Theobroma cacao* L.) germplasm information with domestication history and admixture mapping. Theor Appl Genet 114:877–884

Marri PR, Sarla N, Reddy LV, Siddiq EA (2005) Identification and mapping of yield and yield related QTLs from an Indian accession of *Oryza rufipogon*. BMC Genet 6:33

Matsuoka Y (2011) Evolution of polyploid triticum wheats under cultivation: the role of domestication, natural hybridization and allopolyploid speciation in their diversification. Plant Cell Physiol 52:750–764

Matus I, Corey A, Filichkin T, Hayes PM, Vales MI, Kling J, Riera-Lizarazu O, Sato K, Powell W, Waugh R (2003) Development and characterization of recombinant chromosome substitution lines (RCSLs) using *Hordeum vulgare* subsp *spontaneum* as a source of donor alleles in a *Hordeum vulgare* subsp *vulgare* background. Genome 46:1010–1023

McCouch S (2004) Diversifying selection in plant breeding. PLoS Biol 2(10):e347, doi:10.1371/journal.pbio.0020347

McFadden H, Beasley D, Brubaker CL (2004) Assessment of *Gossypium sturtianum* and G. australe as potential sources of *Fusarium* wilt resistance to cotton. Euphytica 138:61–72

Méndez AM, Castillo D, del Pozo A, Matus I, Morcuende R (2011) Differences in stem soluble carbohydrate contents among recombinant chromosome substitution lines (RCSLs) of barley under drought in a mediterranean-type environment. Agron Res 9((Special Issue II)):433–438

Mujeeb-Kazi A, Rajaram S (2002) Transferring alien genes from related species and genera for wheat improvement. In: Curtis BC, Rajaram S, Gomez Macpherson H (eds.) Bread wheat improvement and production. FAO, Rome, pp 199–215.

Murray TD, De La Peña RC, Yildirim A, Jones SS (1994) A new source of resistance to *Pseudocercosporella herpotrichoides herpotrichoides* cause of eyespot disease of wheat located on chromosome 4V of *Dasypyrum villosum*. Plant Breed 113:281–286

Narasimhamoorthy B, Gill BS, Fritz AK, Nelson JC, Brown-Guedira GL (2006) Advanced backcross QTL analysis of a hard winter wheat × synthetic wheat population. Theor Appl Genet 112:787–796

Naz AA, Kunert A, Lind V, Pillen K, Léon J (2008) AB-QTL analysis in winter wheat: II. Genetic analysis of seedling and field resistance against leaf rust in a wheat advanced backcross population. Theor Appl Genet 116:1095–1104

Neelam K, Rawat N, Tiwari VK, Malik S, Tripathi SK, Randhawa GS, Dhaliwal HS (2011) Molecular and cytological characterization of high grain iron and zinc wheat *Aegilops peregrina* derivatives. Mol Breed 28:623–634

Niu Z, Klindworth DL, Friesen TL, Chao S, Jin Y, Cai X, Xu SS (2011) Targeted introgression of a wheat stem rust resistance gene by DNA marker-assisted chromosome engineering. Genetics 187:1011–1021

Oka HI (1958) Varietal variation and classification of cultivated rice. Ind J Genet Plant Breed 18:78–79

Ortiz R, Braun HJ, Crossa J, Crouch J, Davenport G, Dixon J, Dreisigacker S, Duveiller E, He Z, Huerta J, Joshi AK, Kishii M, Kosina P, Manes Y, Ezzalama M, Morgounov A, Murakami J, Nicol J, Ferrara GO, Ortiz-Monasterio JI, Payne TS, Peña RJ, Reynolds MP, Sayre KD, Sharma RC, Singh RP, Wang J, Warburton M, Wu H, Iwanaga HM (2008) Wheat genetic resources enhancement by the International Maize and Wheat Improvement Center (CIMMYT). Genet Resour Crop Evol 55:1095–1140

Peleman JD, van der Voort JR (2003) Breeding by design. Trends Plant Sci 8:330–334

Pienaar RV (1990) Wheat and *Thinopyrum* hybrids. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 13. Springer, Berlin, pp 167–217

Pillen K, Zacharias A, Léon J (2003) Advanced backcross QTL analysis in barley (*Hordeum vulgare* L.). Theor Appl Genet 107:340–352

Pillen K, Zacharias A, Léon J (2004) Comparative AB-QTL analysis in barley using a single exotic donor of *Hordeum vulgare ssp. spontaneum*. Theor Appl Genet 108:1591–1601

Placido DF, Campbell MT, Jin J, Cui X, Kruger GR, Baenziger PS, Walia H (2013) Introgression of novel traits from a wild wheat relative improves drought adaptation in wheat (*Triticum aestivum*). Plant Physiol. doi:10.1104/pp. 113.214262

Poland JA, Rife TW (2012) Genotyping-by-sequencing for plant breeding and genetics. Plant Genome 5:92–102

Qi Z, Du P, Qian B, Zhuang L, Chen H, Chen T, Shen J, Guo J, Feng Y, Pei Z (2010) Characterization of a wheat-*Thinopyrum bessarabicum* (T2JS-2BS.2BL) translocation line. Theor Appl Genet 121:589–597

Rajaram S, Mann CE, Ortiz-Ferrara G, Mujeeb-Kazi A (1983) Adaptation, stability and high yield potential of certain 1B/1R CIMMYT wheats. In: Sakamoto S (ed) Proceedings of the 6th international wheat genetic symposium, Kyoto, Japan, pp 613–621.

Rajaram S, Villareal RL, Mujeeb-Kazi A (1990) Global impact of 1B/1R spring wheats. In: Agronomy abstracts. ASA, Madison, WI

Rawat N, Tiwari VK, Neelam K, Randhawa GS, Friebe B, Gill BS, Dhaliwal HS (2011) Development and molecular characterization of wheat-*Aegilops kotschyi* addition and substitution lines with high grain protein, iron and zinc. Genome 54:943–953

Rawat N, Tiwari VK, Neelam K, Randhawa GS, Singh K, Chhuneja P, Dhaliwal HS (2009a) Development and characterization of wheat-*Aegilops kotschyi* amphiploids with high grain iron and zinc. Plant Genet Resour 7:271–280

Rawat N, Tiwari VK, Singh N, Randhawa GS, Singh K, Chhuneja P, Dhaliwal HS (2009b) Evaluation and utilization of *Aegilops* and wild *Triticum* species for enhancing iron and zinc content in wheat. Genet Resour Crop Evol 56:53–64

Rawat N, Neelam K, Tiwari VK, Dhaliwal HS (2013) Biofortification of cereals to overcome hidden hunger. Plant Breed. doi: 10.1111/pbr.12040

Rahman ML, Jiang W, Chu SH, Qiao Y, Ham TH, Woo MO, Lee J, Khanam MS, Chin JH, Jeung JU, Brar DS, Jena KK, Koh HJ (2009) High-resolution mapping of two rice brown planthopper resistance genes, *Bph20*(t) and *Bph21*(t), originating from *Oryza minuta*. Theor Appl Genet 119:1237–1246

Reich D, Patterson N (2005) Will admixture mapping work to find disease genes? Phil Trans R Soc B 360:1605–1607

Reynolds MP, Calderini DF, Condon AG, Rajaram S (2001) Physiological basis of yield gains in wheat associated with the *Lr19* translocation from *Agropyron elongatum*. Euphytica 119:137–141

Ribeiro-Carvalho C, Guedes-Pinto H, Harrison G, Heslop-Harrison JS (1997) Wheat-rye chromosome translocations involving small terminal and intercalary rye chromosome segments in the Portuguese wheat landrace Barbela. Heredity 78:539–546

Rieseberg LH, Buerkle C (2002) Genetic mapping in hybrid zone. Am Nat 159:S37–S49

Rieseberg LH, Whitton J, Gardner K (1999) Hybrid zone and the genetic architecture of a barrier to gene flow between two sunflower specie. Genetics 152:713–727

Rustgi S, Shafqat MN, Kumar N, Baenziger PS, Ali ML et al (2013) Genetic dissection of yield and its component traits using high-density composite map of wheat chromosome 3A: Bridging gaps between QTLs and underlying genes. PLoS ONE 8(7): e70526

Saal B, Korff M, Léon J, Pillen K (2011) Advanced-backcross QTL analysis in spring barley: IV. Localization of QTL × nitrogen interaction effects for yield-related traits. Euphytica 177: 223–239

Sacco A, Matteo AD, Lombardi N, Trotta N, Punzo B, Mari A, Barone A (2013) Quantitative trait loci pyramiding for fruit quality traits in tomato. Mol Breed 31:217–222

Salamini F, Özkan H, Brandolini A, Schäfer-Pregl R, Martin W (2002) Genetics and geography of wild cereal domestication in the near east. Nat Rev Genet 3:429–441

Sarikamis G, Marquez J, MacCormack R, Bennett RN, Roberts J, Mithen R (2006) High glucosinolate broccoli: a delivery system for sulforaphane. Mol Breed 18:219–228

Schauer N, Semel Y, Roessner U, Gur A, Balbo I, Carrari F, Pleban T, Perez-Melis A, Bruedigam C, Kopka J, Willmitzer L, Zamir D, Fernie AR (2006) Comprehensive metabolic profiling and phenotyping of interspecific introgression lines for tomato improvement. Nat Biotechnol 24:447–454

Schlegel R, Cakmak I, Torun B, Eker S, Tolay I, Ekiz H, Kalayci M, Braun HJ (1997) Screening for zinc efficiency among wheat relatives and their utilisation for alien gene transfer. In: Braun et al. HJ (eds) Wheat: prospects for global improvement. Proceedings of the 5th international wheat conference, 10–14 June 1996, Ankara, Turkey, pp 347–352

Schmalenbach I, Körber N, Pillen K (2008) Selecting a set of wild barley introgression lines and verification of QTL effects for resistance to powdery mildew and leaf rust. Theor Appl Genet 117:1093–1106

Schmalenbach I, Léon J, Pillen K (2009) Identification and verification of QTLs for agronomic traits using wild barley introgression lines. Theor Appl Genet 118:483–497

Sears ER (1953) Nullisomic analysis in common wheat. Am Nat 87:245–252

Sears ER (1956) The transfer of leaf rust resistance from *Aegilops umbellulata* to wheat. Brookhaven Symposium Biol 9:1–22

Sears ER (1972) Chromosome engineering in wheat. Stadler Symposium 4:23–38

Seifollah K, Alina A, Eduard A (2013) Application of next-generation sequencing technologies for genetic diversity analysis in cereals. In: Gupta PK, Varshney RK (eds) Cereal genomics-II. Springer, New York, pp 77–99

Septiningsih EM, Prasetiyono J, Lubis E, Tai TH, Tjubaryat T, Moeljopawiro S, McCouch SR (2003a) Identification of quantitative trait loci for yield and yield components in an advanced backcross population derived from the *Oryza sativa* variety IR64 and the wild relative *O. rufipogon*. Theor Appl Genet 107:1419–1432

Septiningsih EM, Trijatmiko KR, Moeljopawiro S, McCouch SR (2003b) Identification of quantitative trait loci for grain quality in an advanced backcross population derived from the *Oryza sativa* variety IR64 and the wild relative *O. rufipogon*. Theor Appl Genet 107:1433–1441

Sharma S, Bhat PR, Ehdaie B, Close TJ, Lukaszewski AJ, Waines JG (2009) Integrated genetic map and genetic analysis of a region associated with root traits on the short arm of rye chromosome 1 in bread wheat. Theor Appl Genet 119:783–793

Sharma S, Xu S, Ehdaie B, Hoops A, Close TJ, Lukaszewski AJ, Waines JG (2011) Dissection of QTL effects for root traits using a chromosome arm-specific mapping population in bread wheat. Theor Appl Genet 122:759–769

Shen Y, Shen J, Dawadondup Zhuang L, Wang Y, Pu J, Feng Y, Chu C, Wang X, Qi Z (2013) Physical localization of a novel blue-grained gene derived from *Thinopyrum bessarabicum*. Mol Breed 31:195–204

Sheng H, See DR, Murray TD (2012) Mapping QTL for resistance to eyespot of wheat in *Aegilops longissima*. Theor Appl Genet 125:355–366

Shewry PR, Halford NG (2002) Cereal seed storage proteins: structures, properties and role in grain utilization. J Exp Bot 53:947–958

Simmonds J, Fish L, Leverington-Waite M, Wang Y, Howell P, Snape JW (2008) Mapping of a gene (*Vir*) for a non-glaucous, viridescent phenotype in bread wheat derived from *Triticum dicoccoides*, and its association with yield variation. Euphytica 159:333–341

Singh RJ (2007) Landmark research in oilseed crops. In: Singh RJ (ed) Genetic resources, chromosome engineering, and crop improvement: oilseed crops. CRC Press, Boca Raton, FL, pp 1–12

Singh RJ, Hymowitz T (1999) Soybean genetic resources and crop improvement. Genome 42:605–616

Singh RP, Hodson DP, Huerta-Espino J, Jin Y, Njau P, Wanyera R, Herrera-Foessel SA, Ward RW (2008) Will stem rust destroy the world's wheat crop? Adv Agron 98:271–309

Snape JW, Foulkes MJ, Simmonds J, Leverington M, Fish LJ, Wang Y, Ciavarrella M (2007) Dissecting gene × environmental effects on wheat yields via QTL and physiological analysis. Euphytica 154:401–408

Song WY, Wang GL, Chen LL, Kim HS, Pi LY, Holsten T, Gardner J, Wang B, Zhai WX, Zhu LH, Fauquet C, Ronald P (1995) A receptor kinaselike protein encoded by the rice disease resistance gene, *Xa21*. Science 270:1804–1806

Stevens R, Buret M, Duffé P, Garchery C, Baldet P, Rothan C, Causse M (2007) Candidate genes and quantitative trait loci affecting fruit ascorbic acid content in three tomato populations. Plant Physiol 143:1943–1953

Stewart J McD (1995) Potential for crop improvement with exotic germplasm and genetic engineering. In: Git C, Forrester NW (eds) Challenging the future, proceeding of world cotton research conference. CSIRO, Melbourne, pp 313–327.

Stokstad K (2007) Deadly wheat fungus threatens world's breadbaskets. Science 315:1786–1787

Suslow TV, Thomas BR, Bradford KJ (2002) Biotechnology provides new tools for plant breeding. Agr Biotechnol Calif Ser Publ 8043:1–19

Swaminathan MS (2009) Gene banks for a warming planet. Science 325:517

Swaminathan MS (2010) Achieving food security in times of crisis. New Biotechnol 27:453–460

Tanksley SD, Grandillo S, Fulton TM, Zamir D, Eshed Y, Petiard V, Lopez J, Beck-Bunn T (1996) Advanced backcross QTL analysis in a cross between an elite processing line of tomato and its wild relative *L. pimpinellifolium*. Theor Appl Genet 92:213–224

Tanksley SD, Nelson JC (1996) Advanced backcross QTL analysis: a method for the simultaneous discovery and transfer of valuable QTLs from unadapted germplasm into elite breeding lines. Theor Appl Genet 92:191–203

Thomson MJ, Tai TH, McClung AM, Lai XH, Hinga EM, Lobos KB, Xu Y, Martinez CP, McCouch SR (2003) Mapping quantitative trait loci for yield, yield components and morphological traits in an advanced backcross population between *Oryza rupogon* and the *Oryza sativa* cultivar Jefferson. Theor Appl Genet 107:479–493

Tian F, Li DJ, Fu Q, Zhu ZF, Fu YC, Wang XK, Sun CQ (2006) Construction of introgression lines carrying wild rice (*Oryza rufipogon* Griff.) segments in cultivated rice (*O. sativa* L.) background associated with yield-related traits. Theor Appl Genet 112:570–580

Timonova EM, Leonova IN, Roeder MS, Salina EA (2013) Marker-assisted development and characterization of a set of *Triticum aestivum* lines carrying different introgressions from the *T. timopheevii* genome. Mol Breed 31:123–136

Tiwari VK, Rawat N, Neelam K, Kumar S, Randhawa GS, Dhaliwal HS (2010) Substitution of 2S and 7U chromosomes of *Aegilops kotschyi* in wheat enhances grain iron and zinc concentration. Theor Appl Genet 121:259–269

Tonguc M, Griffiths PD (2004) Transfer of powdery mildew resistance from *Brassica carinata* to *Brassica oleracea* through embryo rescue. Plant Breed 123:587–589

Trethowan RM, Mujeeb-Kazi A (2008) Novel germplasm resources for improving environmental stress tolerance of hexaploid wheat. Crop Sci 48:1255–1265

Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J (2006) A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat. Science 314:1298–1301

Veasey EA, Karasawa PP, Santos MS, Rosa E, Mamani E, Oliveira CX (2004) Variation in the loss of seed dormancy during after-ripening of wild and cultivated rice species. Ann Bot 94:875–882

Villareal RL, Del-Toro E, Mujeeb-Kazi A, Rajaram S (1995) The 1BL/1RS chromosome translocation effect on yield characteristic in a *Triticum aestivum* L. cross. Plant Breed 14:497–500

von Bothmer R, Jacobsen N, Baden C, Jargensen RB, Linde-Laursen I (1995) An ecogeographical study of the genus *Hordeum*. 2nd Edn., International Plant Genetic Resources, Rome

von Korff M, Wang H, Léon J, Pillen K (2004) Development of candidate introgression lines using an exotic barley accession (*Hordeum vulgare ssp. spontaneum*) as donor. Theor Appl Genet 109:1736–1745

von Korff M, Wang H, Léon J, Pillen K (2005) AB-QTL analysis in spring barley. I. Detection of resistance genes against powdery mildew, leaf rust and scald introgressed from wild barley. Theor Appl Genet 111:583–590

von Korff M, Wang H, Léon J, Pillen K (2006) AB-QTL analysis in spring barley: II. Detection of favourable exotic alleles for agronomic traits introgressed from wild barley (*H. vulgare* ssp. *spontaneum*). Theor Appl Genet 112:1221–1231

von Korff M, Wang H, Léon J, Pillen K (2008) AB-QTL analysis in spring barley: III. Identification of exotic alleles for the improvement of malting quality in spring barley (*H. vulgare* ssp. *spontaneum*). Mol Breed 21:81–93

Vreugdenhil D, Aarts MGM, Koorneef M (2005) Exploring natural genetic variation to improve plant nutrient content. In: Broadley MR, White PJ (eds) Plant nutritional genomics. Blackwell, Oxford, UK, pp 201–219

Wang B, Yichun N, Zhongxu L, Xianlong Z, Junjie L, Jing B (2012) Molecular diversity, genomic constitution, and QTL mapping of fiber quality by mapped SSRs in introgression lines derived from *Gossypium hirsutum* × G. darwinii Watt. Theor Appl Genet 125:1263–1274

Wang B, Chee PW (2010) Application of advanced backcross quantitative trait locus (QTL) analysis in crop improvement. J Plant Breed Crop Sci 2:221–232

Wang L, Xu C, Qu M, Zhang J (2008a) Kernel amino acid composition and protein content of introgression lines from *Zea mays* ssp. *mexicana* into cultivated maize. J Cereal Sci 48:387–393

Wang L, Yang A, He C, Qu M, Zhang J (2008b) Creation of new maize germplasm using alien introgression from *Zea mays* ssp. *mexicana*. Euphytica 164:789–801

Wang RRC, Larson SR, Horton WH, Chatterton NJ (2003) Registration of W4909 and W4910 bread wheat germplasm lines with high salinity tolerance. Crop Sci 43:746

Wang S, Yin L, Tanaka H, Tanaka K, Tsujimoto H (2011) Wheat-*Aegilops* chromosome addition lines showing high iron and zinc contents in grains. Breed Sci 61:189–195

Welch RM, Graham RD (2002) Breeding crops for enhanced micronutrient content. Plant Soil 245:205–214

Welch RM, Graham RD (2004) Breeding for micronutrients in staple food crops from a human nutrition perspective. J Exp Bot 55:353–364

Wendel JF, Cronn RC (2003) Polyploidy and the evolutionary history of cotton. Adv Agron 78:139–186

White PJ, Broadley MR (2009) Biofortification of crops with seven mineral elements often lacking in human diets—iron, zinc, copper, calcium, magnesium, selenium and iodine. New Phytol 182:49–84

Wu J, Yang X, Wang H, Li H, Li L, Li X, Liu W (2006) The introgression of chromosome 6P specifying for increased numbers of florets and kernels from *Agropyron cristatum* into wheat. Theor Appl Genet 114:13–20

Wu JL, Sinha PK, Variar M, Zheng KL, Leach JE, Courtois B, Leung H (2004) Association between molecular markers and blast resistance in an advanced backcross population of rice. Theor Appl Genet 108:1024–1032

Xiao J, Li J, Grandillo S, Ahn SN, Yuan L, Tanksley SD, McCouch SR (1998) Identification of trait-improving quantitative trait loci alleles from a wild rice relative, *Oryza rufipogon*. Genetics 150:899–909

Xiao J, Li J, Yuan L, McChouch SR, Tanksley SD (1996) Genetic diversity and its relationship to hybrid performance and heterosis in rice as revealed by PCR-based markers. Theor Appl Genet 92:637–643

Xie X, Jin F, Song MH, Suh JP, Hwang HG, Kim YG, McCouch SR, Ahn SN (2008) Fine mapping of a yield-enhancing QTL cluster associated with transgressive variation in an *Oryza sativa* × *O. rufipogon* cross. Theor Appl Genet 116:613–622

Yamamoto T, Lin H, Sasaki T, Yano M (2000) Identification of heading date quantitative trait locus *Hd6* and characterization of its epistatic interactions with *Hd2* in rice using advanced backcross progeny. Genetics 154:885–891

Yano M (2001) Genetic and molecular dissection of naturally occurring variation. Curr Opin Plant Biol 4:130–135

Yoon DB, Kang KH, Kim HJ, Ju HG, Kwon SJ, Suh JP, Jeong OY, Ahn SN (2006) Mapping quantitative trait loci for yield components and morphological traits in an advanced backcross population between *Oryza grandiglumis* and the *O. sativa japonica* cultivar Hwaseongbyeo. Theor Appl Genet 112:1052–1062

Yoshimura A, Nagayama H, Sobrizal, Kurakazu T, Sanchez PL, Doi K, Yamagata Y, Yasui H (2010) Introgression lines of rice (*Oryza sativa L.*) carrying a donor genome from the wild species, *O. glumaepatula* Steud. and *O. meridionalis* Ng. Breed Sci 60:597–603

Yu F, Lydiate DJ, Gugel RK, Sharpe AG, Rimmer SR (2012) Introgression of *Brassica rapa* subsp. *sylvestris* blackleg resistance into *B. napus*. Mol Breed 30:1495–1506

Yu J, Holland JB, McMullen MD, Buckler ES (2008) Power analysis of an integrated mapping strategy: nested association mapping. Genetics 138:539–551

Yun SJ, Gyenis L, Bossolini E, Hayes PM, Matus I, Smith KP, Steffenson BJ, Tuberosa R, Muehlbauer GJ (2006) Validation of quantitative trait loci for multiple disease resistance in barley using advanced backcross lines developed with a wild barley. Crop Sci 46:1179–1186

Zaharieva M, Gaulin E, Havaux M, Acevedo E, Monneveux P (2001) Drought and heat responses in the wild wheat relative *Aegilops geniculata* Roth: potential interest for wheat improvement. Crop Sci 41:1321–1329

Zamir D (2001) Improving plant breeding with exotic genetic libraries. Nat Rev Genet 2:983–989

Zan-Min H (2003) Salt tolerance transferred from wild Triticeae species into wheat. Inform Syst Biotechnol. Feb 2003 (http://www.isb.vt.edu; verified 24 Apr 2008).

Zeven AC (1980) Polyploidy and domestication: the origin and survival of polyploids in cytotype mixtures. In: Lewis WH (ed) Polyploidy: basic life sciences, vol 13. Springer, FL, USA, pp 385–407

Zhang R, Wang X, Chen P (2012) Molecular and cytogenetic characterization of a small alien-segment translocation line carrying the softness genes of *Haynaldia villosa*. Genome 55:639–646

Chapter 10
Gene Flow and Risk Assessment in Genetically Modified Crops

Stephen F. Chandler and Trevor W. Stevenson

Abstract For developers and regulators of genetically modified plants, an evaluation of gene flow is an essential part of the risk assessment required prior to a decision on whether a transgenic crop variety may enter the marketplace. Assessments of the probability of gene flow relative to non-GM varieties and the potential impact of the establishment of a transgene in populations outside of cultivation are used to determine a level of risk. We provide here an overview of the principles and methodologies which have been used in gene flow studies in transgenic crops.

Keywords Gene dispersal • GM crops • Transgene • Herbicide resistance • Gene flow impact

10.1 Introduction

Since the introduction of transgenic, or genetically modified (hereafter abbreviated to "GM") crops, the potential for gene flow has been a central consideration for those developing, commercialising and regulating GM crops. In assessing potential impacts of gene flow, it is necessary to know what the probability of gene flow to, from and within the crop under consideration is and what is the likely impact of "escape" of transgene(s) from the cultivated environment (Glover 2002; Gomez-Galera et al. 2012). This may be considered through an examination of the components of risk assessment; the probability of an event happening and the possible

S.F. Chandler • T.W. Stevenson, Ph.D. (✉)
School of Applied Sciences, RMIT University, Melbourne, VIC 3083, Australia
e-mail: stephen.chandler@rmit.edu.au; trevor@rmit.edu.au

A. Pratap and J. Kumar (eds.), *Alien Gene Transfer in Crop Plants, Volume 1: Innovations, Methods and Risk Assessment*, DOI 10.1007/978-1-4614-8585-8_10,
© Springer Science+Business Media New York 2014

impacts of such an event happening. An assessment of probability comes from baseline knowledge of the crop in question coupled with case-by-case considerations of possible gene flow with the related phenotypic changes in the GMO. The potential impact of the occurrence of an event will very much depend on case-specific factors, such as the introduced trait, the crop or GM species in question, the release site and the eventual use of the crop, the GM plant or derivatives of these. In this short article, it is not possible to review the extensive work that has been carried out in gene flow in transgenic crops, and by no means the breadth of literature available on introgression in plants, or risk assessment applied to non-transgenic invasive plant species (Pysek 2001; Lee 2002). More detailed information and viewpoints on this aspect have been discussed earlier in several reviews (Becker et al. 1992; Ellstrand et al. 1999; Ellstrand and Schierenbeck 2000; Abbot et al. 2003; Stewart et al. 2003; Gealy et al. 2007; Wilkinson and Ford 2007; Chandler and Dunwell 2008). Besides, general issues on integration of transgene into agricultural plants have been discussed excellently by Ellstrand (2003, 2006) in his reviews. Because weedy relatives exist for most major crops (Gealy et al. 2007), impact of gene flow and introgression from domesticated plants to wild relatives has also been reviewed earlier (Ellstrand et al. 1999). This chapter only provides a practical framework around which to build a plan to consider gene flow issues for those contemplating the development of a transgenic plant product.

10.2 Mechanisms of Gene Flow

The potential routes for gene flow from a transgenic crop are the same as those for conventional non-GM varieties of the same crop. These routes include gene dispersal from the crop via natural mechanisms of seed (Tiffney 2004; Cummings et al. 2008; Bailleul et al. 2012) or pollen (Beckie et al. 2012) dissemination, persistence of feral populations in and around cultivated areas as a result of long-lived vegetative organs or seed banks (Desplanque et al. 1999; Pohl-Orf et al. 1999; Walker et al. 2004), hybridisation with related weed species in and around areas under cultivation, hybridisation of GM to non-GM varieties of the same species. Human-aided dispersal mechanisms are also important. In the case of ornamentals, for example, both plants and flowers are traded at an international level, potentially introducing alien species (Anderson 2007; Cook and Proctor 2007; Aldous et al. 2011).

10.2.1 Baseline Information on Crop Biology

Good knowledge of the fundamental biology of a crop is essential in order to estimate the probability of gene dispersal. For the major crops, there is detailed knowledge of their reproductive and seed biology and consensus documents are published

Table 10.1 Morphological and physiological parameters that can be measured to provide baseline information on gene flow in transgenic plants

Parameter	Variables that can be measured
Reproductive organs	Flowers per plant; number of styles, stamens and viable anthers per flower; size of reproductive organs; dynamics of cross- and self-compatibility
Vegetative reproduction capacity	Number of reproductive organs per plant; percentage adventitious root formation; dormancy
Pollen	Percentage of viable anthers; pollen viability; pollen germination; pollen grain size
Flowering	Number of flowers per plant; flower longevity on plant and detached from plant; flowering time; patterns of flower opening; period of receptivity; rates of successful pollination and seed set; presence and nature of pollination track lines
Seed	Number of seed and fruit formed per plant; seed size and weight; size of seed capsules; strength of seed capsules; seed germination
Persistence	Dormancy/survival times for vegetative organs; pollen longevity; seed dormancy

by a variety of regulatory authorities (Luijten and de Jong 2010), the Organization for Economic Cooperation and Development (OECD) and within the scientific literature (Arriaga et al. 2006; Ferreira et al. 2007; Gealy et al. 2007). For some crops, models of gene flow are now available (Colbach et al. 2001; Baker and Preston 2003; Cresswell 2003; Meagher et al. 2003; Fricke et al. 2004; Glemnitz et al. 2011; Schmidt and Schroder 2011; Tyson et al. 2011). However, for minor crops, a detailed baseline may not be available, but can often be generated in the experimental data that will be required to assess the probability of gene flow from the GM crop under consideration. Where a minor crop has not been known to hybridise with wild relatives, experimental crosses to assess the potential for interspecific reproduction may be appropriate (Armstrong et al. 2005; FitzJohn et al. 2007). In these experiments, transgenic lines can be assessed against comparator lines, including the variety used to initially develop the GM plant under consideration. Data that can be measured for assessment are summarised in Table 10.1. Decisions need to be made on parameters most relevant for assessment, and these parameters need to be based on the biology of the crop. For example, considering a vegetatively propagated horticultural crop, adventitious rooting capacity will be more relevant than a detailed analysis of pollen production. For an insect pollinated crop, factors that may influence the amount of visits per plant by insect vectors will be more relevant. Depending on the preliminary data generated, more specific experiments can be carried out such as measuring a specific parameter like flowering time under a number of environmental conditions. Clearly, these supplementary experiments will be mandatory if the introduced genes confer upon the GM plant a phenotype affecting parameters such as those described in Table 10.1.

Regulators are particularly interested in compatibility with related species, especially if such related species include weeds, as is the case for oilseed rape

Table 10.2 Examples of environmental factors which may affect the probability of gene flow

Factor	Examples of parameters affecting gene flow
Seasonal	Effects of temperature and/or drought on pollinator abundance; wind direction and speed; abundance and type of nearby food sources for insect pollinators; abundance and type of potential vectors for seed dispersal
Wild relatives	Type and frequency; flowering times in comparison to crop; compatibility with related species
Agronomic practices	Normal practice for control of related weed species; normal practice for control of feral species; normal practice for harvest and distribution of seed
Proximity to non-GM varieties	Expected proximity to compatible varieties; synchrony of flowering with non-GM varieties
Importance of variety segregation	Proximity to elite lines; importance of need for variety segregation

(Warwick et al. 2003; FitzJohn et al. 2007) and sugar beet (Desplanque et al. 1999; Cureton et al. 2006). Analysis of the wild flora in and around field trial sites (Chandler et al. 2008) is important in order to eliminate or evaluate the potential for hybridisation to wild-related species.

In addition to reproductive and seed biology, knowledge of the agronomy of a crop is also important from a gene flow perspective. For example, whether the crop typically produces seed banks or feral populations, or whether non-GM conventional plant varieties have historically become weeds outside of cultivation. One benefit of the focus on transgenic crop safety by regulators has been that more is now known about gene flow and some of the previously less quantified mechanisms of gene flow, such as seed spillage (von der Lippe and Kowarik 2007). For herbicide-tolerant transgenic varieties, baseline knowledge should also include gene flow studies with conventionally bred herbicide resistant varieties (Mallory-Smith and Sanchez Olguin 2011; Krato and Petersen 2012; Presotto et al. 2012). Some crops may already be known to regularly hybridise with related species in the wild. Information on the biology of these hybrids and how they evolve (Vacher et al. 2011) should be included in baseline information.

10.2.2 Factors Affecting Gene Flow

Having established good baseline knowledge, it is necessary to identify the major environmental and ecological factors, and their interactions, that can impact gene flow (Becker et al. 1992). Some of the more significant examples are listed in Table 10.2.

For crops that are insect-pollinated, knowledge of insect foraging behaviour (Cresswell 2000; Osborne et al. 1999; Zaller et al. 2007; Tyson et al. 2011) is critical to understand the probability of gene dispersal at varying distance from the crop.

Agronomically, manipulation of the amount and type of transgenic material in a crop may affect gene flow (Feili et al. 2003), as can environmental factors such as carbon dioxide concentration (Ziska et al. 2012). In *Brassica napus*, there is variation in out-crossing rates at different locations (Becker et al. 1992). In sugar beet, habitat appears to dictate the amount of gene flow observed (Cureton et al. 2006).

10.3 Assessing the Probability of Gene Flow

Once the baseline information is in place, this can be complemented with data from laboratory experiments, greenhouse experiments and field trials to provide a measure of the probability of gene flow. Establishing a measure of the probability of gene flow has two purposes: firstly to evaluate the inherent probability for the crop under consideration and secondly to evaluate whether there is any increased or reduced probability of gene flow from the transgenic variety relative to that observed with the conventional non-GM variety it is derived from.

10.3.1 Experimental Data

Side by side experiments in which transgenic lines are assessed against comparator lines will generate data that will be of great assistance in establishing whether there are significant differences between the modified line(s) and conventional, non-GM line(s). Intra-varietal variation in parameters affecting gene flow is likely, particularly for the major crops and any significant differences may be less significant than the variance observed between non-GM varieties of the crop. An understanding of this variation is important when evaluating any differences between the transgenic line and the variety it was derived from.

Experimental design factors such as plot size (Willenborg et al. 2009; Palaudelmas et al. 2012; Rong et al. 2012), sampling error (Begg et al. 2007) and pollen density (Goggi et al. 2007) can have an effect on the measured rates of gene flow. The decision on whether to use gaps or plants in assessing separation distance was observed to have a significant impact on measurements of gene flow in oil seed rape, a species that is both wind and insect pollinated (Reboud 2003).

10.3.2 Field Studies

Experimental data is usually collected from contained experiments where the environment is relatively closely controlled. The advantage of contained experiments is that relatively small differences between lines can be measured. However, many external variables are excluded in such trials and field trials provide additional

information on gene flow. There is no substitution for field trials if pollen flow can be expected across many hundreds of metres, as is the case in canola, for example (Beckie et al. 2003; Devaux et al. 2005). Field trials also provide the opportunity to measure gene flow under natural conditions in which pollen spread is left to the wind (Hoyle and Cresswell 2007) or naturally occurring pollination vectors (Pons et al. 2011). Field trials should be carried out over several seasons if the intention is to understand the affect of seasonal factors such as temperature and rainfall variation on gene flow. In designing experiments to measure the rates of gene flow it is most ideal to have at hand a reliable and inexpensive technique to identify and track the transgene. This is because identification of very low frequencies of gene flow will require the analysis of populations of tens of thousands or even millions (Jhala et al. 2012) of individual samples. Readily identifiable physical tags such as reporter gene (Messeguer et al. 2001; Halfhill et al. 2003; Lim et al. 2007; Pons et al. 2011) or colour modification genes (Tanaka et al. 2009; Kovinich et al. 2012) are very useful in this respect. A real time bioluminescent technique has recently been reported (Kiddle et al. 2012) and real-time PCR has been shown to be a reliable indicator of cross-pollination in maize (Pla et al. 2006). In the absence of suitable markers, analysis of batched samples (Guadagnuolo et al. 2001; Baack 2006; Mazzara et al. 2012) is necessary but when hybridisation events are expected to occur at a very low frequency the level of inaccuracy (false negatives and positives) inherent in a technique must also be quantified (Peter et al. 2001; Begg et al. 2007). Where a crop produces a large number of seed a minimum number of seed must be determined for analysis in order for statistical analysis to be applied (Jhala et al. 2012). In some cases gene flow must be measured in the absence of a phenotype. For example, the introduced gene may only be expressed in certain tissues, or may be transferred, but not expressed, in recipient genetic backgrounds. In these cases molecular techniques are used to detect the presence of introduced genes (Abbot et al. 2003; Nakamura et al. 2011).

Estimation of segregation distance generates important data for segregation rules (for example separating organic and non-organic production, or varieties which are being grown for non-food use). For trees (DiFazio et al. 2004; Pons et al. 2011) and some ornamental crops (Gross et al. 2012) long-term field studies are required to collect data on gene flow (Smouse et al. 2007). This is because of the long time to maturation and the annual cycle of pollen and seed formation. The seed may also have a long dormancy period—in these cases experiments in which dormancy breaking methods are compared for the transgenic line and the parent are most useful. One of the first transgenic crops to be commercialised was *Brassica napus* (oilseed rape, canola). Gene flow from this species is mediated by both pollen (Lavigne et al. 1998; Beckie et al. 2003; Funk et al. 2006) and seed dispersal (Lutman et al. 2005; Devos et al. 2012) and has been evaluated on a landscape scale in the UK (Weekes et al. 2005) and the US (Schafer et al. 2011). Like other species (Luo et al. 2005; Fenart et al. 2007) oilseed rape pollen can be dispersed over long distances (Devaux et al. 2005). For species such as *Brassica* and wind pollinated grasses (Wang et al. 2004) experimental design and workload for accurate measurement of separation distance is obviously a challenge. Nevertheless, crop-crop level

Table 10.3 Experimental measurements of gene flow from transgenic crops

Crop	Reference
Soybean (*Glycine max*)	Abud et al. (2007)
Oilseed rape, canola (*Brassica napus*)	Lavigne et al. (1998), Beckie et al. (2003), Reboud (2003)
Bahiagrass (*Paspalum notatum*)	Sandhu et al. (2009, 2010)
Wheat (*Triticum aestivum*)	Brule´-Babel et al. (2006), Gatford et al. (2006), Willenborg et al. (2009)
Chinese cabbage (*Brassica rapa* ssp. *pekinensis*)	Lim et al. (2007)
Maize (*Zea mays*)	Pla et al. (2006), Weekes et al. (2007), Viljoen and Chetty (2011)
Flax (*Linum usitatissimum*	Jhala et al. (2012)
Potato (*Solanum tuberosum*)	Scurrah et al.(2008), Bravo-Segretin et al. (2011)
Barley (*Hordeum vulgare*)	Gatford et al. (2006)
Rice (*Oryza sativa*)	Messeguer et al. (2001, 2004), Chun et al. (2011), Rong et al. (2012)
Tomato (*Lycopersicon esculentum*)	Ilardi and Barba (2002)
Sugar beet (*Beta vulgaris*)	Pohl-Orf et al. (1999)
Citrus sp.	Pons et al. (2011)

gene flow (Weekes et al. 2007; Rieger et al. 2007) is the reality of the agricultural situation and there are examples where gene flow has been estimated for a crop on a territory basis (Wilkinson et al. 2000, 2003; Züghart 2010; Sausse et al. 2012). Field studies can also be extended to the study of already established feral populations, as have occurred from seed spillage in *Brassica napus* (Claessen et al. 2005). Examples of experimental research on gene flow in transgenic crops are tabulated in Table 10.3.

10.3.3 Tools to Reduce the Probability of Gene Flow

Physical barriers to gene flow are usually mandatory in the early stages of evaluation of transgenic lines (Glaser 2003; van Hengstum et al. 2012). Typically these small-scale trials are carried out in contained conditions in which the potential for insect pollination is minimised. In the field, trials can be physically isolated from any potential recipient plants by distance or by suitable inclusion of gaps (Reboud 2003) and/or barrier plants. More permanent genetic modification techniques to reduce the probability of gene flow have been proposed, including Barnase-based systems (Lannenpaa et al. 2005; Kobayashi et al. 2006), "gene deletor" technology (Li 2012), selection of varieties with low inherent potential for gene flow (Gruber et al. 2012; Ohmori et al. 2012) or inclusion of genes which will allow feral plants to be killed by chemicals (Liu et al. 2012). Whilst we will not review these techniques in detail here (see review Chapman and Burke 2006 for details on this subject), clearly these are examples of where the probability of gene flow is affected by

expression of the transgene (Glaser 2003). Depending on the crop, regulators may ask for significant evidence that this reduced probability is not conditional, i.e., is expressed in the range of environmental and agronomic conditions that the transgenic plant is likely to be grown in. Transgene mitigation has been proposed as a tool to reduce the fitness of any escaped genes, on the assumption the transgenic trait will escape (Al-Ahmad et al. 2005).

10.3.4 Quantification of Gene Flow

The most common example of quantification of gene flow is the establishment of segregation distance, based on hybridisation from outside the crop of interest. This is usually quantified by measuring the occurrence of transgenic/non-transgenic hybrid seeds at varying distances from a centrally located plot of transgenic plants, surrounded by non-transgenic plants (Lavigne et al. 1998; Beckie et al. 2003; Funk et al. 2006; Gatford et al. 2006; Pla et al. 2006; Abud et al. 2007; Jhala et al. 2012). It is difficult to provide an assessment of a probability when that probability is very close to zero and the crop in question has no history of establishing as a feral population or of introgression to other related species. If a transgenic trait does confer a competitive advantage, even at a very low frequency of gene transfer this trait may eventually become incorporated into and subsequently maintained in populations of wild or related species. In these cases, it is more useful to focus on any differences between the transgenic line and non-GM varieties of the same species. It is reasonable to assume the characteristics of the crop will also apply to the transgenic. For example, if there is a history of feral population establishment for a crop, this is also likely to occur for the transgenic varieties. Likewise, if a crop has no history of introgression or invasiveness, this is also likely to be the case for the transgenic. Now there is a history of cultivation of transgenic crops, the weight of data indicates that the gene flow characteristics of transgenic varieties do, to a large extent, mirror those of non-GM varieties of the same species.

10.4 Assessing the Impact of Gene Flow

Assuming gene flow is probable and that the most likely mechanism of gene flow has been identified, the impact of gene flow can be determined. This impact will almost certainly focus on any phenotype caused by expression of the transgene and will be again need to be assessed in terms of whether the impact is likely to be greater or less than that imposed by gene flow from comparable non-GM plants of the same species. Where possible, comparison with similar phenotypes such as for GM herbicide-tolerant varieties and non-GM herbicide-resistant varieties is appropriate. The impact may or may not present a hazard (Gealy et al. 2007). Relevant questions will focus on whether the expression of the transgene in the absence of

selection pressure confers any competitive advantage. The complexity of this type of assessment has been increased with the introduction of stacked genes (Orson 2002; Liu et al. 2012) in single GM varieties. The inserted transgenes may, for example, confer both herbicide resistance and insect resistance phenotypes to an individual plant (Lenaic et al. 2012). Where there is the possibility of transfer of several different transgenes, specific molecular analysis methods are required in order to assess the gene flow frequencies of each gene (Wang et al. 2012).

10.4.1 Gene Flow in Commercial Transgenic Crops

For some transgenic crop/trait combinations there is now an extended history of production, over massive areas. In North America, herbicide-tolerant and insect-resistant varieties of upland cotton, soybean and maize now dominate for these three crops representing 94, 93 and 88 %, respectively, of all plantings in 2012 (National Agricultural Statistics Service, Agricultural Statistics Board, United States Department of Agriculture, June 29 2012). With this history and the enormous scale of planting an understanding of the potential for gene flow to occur, at least in the North American environment, has moved from theoretical to an actual understanding. Raybould et al. (2012) have assessed experimentally the potential ecological risks from feral populations of insect-resistant transgenic maize, concluding transgenic maize posed a similar invasiveness potential to non-transgenic maize.

10.4.2 Experimental Studies

Several experiments have been designed to assess whether the expression of transgenes confers any form of selective advantage to transgenic plants in comparison to other varieties or in cultivated competition with comparable varieties (Al-Ahmad et al. 2005; Chapman and Burke 2006; Burke and Riesenberg 2007; Yang et al. 2012). Such information is critical to assessing the potential impact of gene flow. Introduction of a transgene may come at a metabolic cost, reducing plant fitness (Tardif et al. 2006) and thereby reducing potential competitiveness. However, more direct experiments and observations to test whether transgenes will survive when introduced into the environment are needed. Warwick et al. (2007) generated transgenic sunflowers carrying a disease resistance gene. Hybrids were then generated with related non-GM varieties and subjected to selection pressure (Burke and Riesenberg 2007). To be informative, measurement of fitness under experimental conditions requires replication and repetition in a variety of different environments and conditions, and ideally should be conducted in several different genetic backgrounds (Burke and Riesenberg 2007). Transgenic herbicide-tolerant oilseed rape has escaped from cultivation via seed spillage, presenting an opportunity to introduce the tolerance to weeds, but Devos et al. (2012) have stated the trait would only

be amplified if the herbicides to which herbicide-tolerant volunteers are tolerant were used routinely in the field. The performance of direct field plot experiments to measure the potential invasiveness of the GM volunteers compared to the non-GM parents, as has been done for insect-resistant maize (Raybould et al. 2012) would be valuable. In canola the herbicide tolerance trait did become established in weedy relatives in the absence of selection pressure (Dlugosch and Whitton 2008). However, in the case of the maize there was no increase in invasiveness observed (Raybould et al. 2012). Yang et al. (2012) evaluated the fitness advantage of the *Bt* gene in a hybrid developed between transgenic and weedy rice, concluding the fitness of insect resistance would be unlikely to spread from transgenic rice crops to related weeds growing nearby.

10.5 Risk Assessment

Literature reviews, field studies and larger scale releases will all generate information upon which it will be possible to estimate a probability of gene flow and generate an estimate of the magnitude of this gene flow under different environmental conditions. As explained above, this probability will need to be placed into the context of conventional varieties. It is important to know whether the transgenic line has any characteristics that either reduce or increase the probability of gene flow. Once a probability is determined then together with the potential impact these then provide a basis to estimate risk. As this depends on phenotype conferred by the introduced gene; the phenotype or phenotypes generated by this gene or genes, the species, and in some cases the specific varieties under consideration, this assessment is nearly always on a case-by-case basis.

10.5.1 Quantification of Risk

A variety of frameworks exist that quantify risk and uncertainty in the context of gene flow. However, such frameworks must be adapted to the precise crop/trait combination under consideration, making the quantification of risk complex and by extension imprecise. For example, more emphasis is likely to be placed on non-food transgenic lines compatible with food varieties of the same species (for example, corn) if those non-food lines produce pharmaceuticals or are designed for biofuels (Kausch et al. 2010; Wang and Brummer 2012). Evaluation of gene flow issues related to the production of pharmaceuticals, industrial compounds and/or biofuels in GM lines of non-food plant species is likely to be more focused on the possibility of hybridisation to related wild species.

As mentioned earlier, there has been significant experience with commercial transgenic crops planted over very wide areas in North America and this has added to knowledge on the probability and actual extent of gene flow from these particular

plant species. However, despite provision of this observational data, most risk assessment processes remain qualitative, assessing risk on scales of probability (low to high) or impact (none to hazardous). Quantitative assessment of risk is very difficult for a commercially transgenic crop, which will be grown in numerous environments under climatic conditions which can not be precisely predicted. All of the information gathered during collection of baseline information, together with experimental data are used to estimate risk (refer to Gealy et al. 2007 for an overview). Where the probability of dispersal of the transgene is certain (Dlugosch and Whitton 2008) much more attention will need to be paid to the potential impact of the effect of expression of the inserted genes.

10.5.2 Post Release Monitoring

Post release monitoring, which is mandatory as part of the release of certain GMOs and in certain territories (Pascher et al. 2011; Züghart et al. 2011) can provide useful information supplementing the suppositions on the rate of gene flow (Sanvido et al. 2006, 2007, 2008). Post release monitoring provides an opportunity to review the accuracy and validity of risk assessments performed prior to the release of the transgenics and is also essential where there exists potential for long-term impact on the ecosystem (Heinemann and El-Kawy 2012). For example, whether herbicide resistance or pest resistance is likely to become integrated into the gene pool of weed species (Wozniak and Martinez 2011) or whether pests and weeds may evolve resistance. Herbicide resistance as a trait, whether developed by GM technologies or conventional breeding or mutagenesis will quickly establish in weed populations if those weeds are in or adjacent to production of herbicide-tolerant varieties, and are so regularly subjected to selection pressure (Brule´-Babel et al. 2006). General surveillance monitoring via floristic databases and vegetation surveys (Chandler et al. 2008) can be used to confirm lack of establishment of the GMO to hybrids outside of cultivation. Detailed surveys directed at the borders or within the production areas are an ideal test environment, as this is where the probability of hybridisation and feral population establishment is greatest. Case-specific monitoring in comparison to general monitoring has been reviewed by Heinemann and El-Kawy (2012). It is important to note that post release monitoring has so far not lead to the recall of any transgenic plant variety. However, the information can be useful—should the recall of any very widely grown product be required its removal from the environment once marketing has stopped and farmers stop growing saved seed will rely on the information on gene flow gathered during development and production to identify the mechanisms by which the gene could have dispersed. These avenues can then be followed to remove the genes from the environment. Continual monitoring is also valuable given that regulators need to evaluate the probability and risks of gene flow in the ever changing environment of an agricultural situation. The effects of climate change, for example, may well change the dynamics of gene flow in the future as suggested by Ziska et al. (2012).

10.6 Conclusions

In the end, because the risk assessment will always come down to a case-by-case basis, it is difficult to provide a simple and widely applicable formula which a developer or a regulator can apply to whether a particular transgenic product will pose an unacceptable risk. From a purely scientific perspective there are those that argue the risks associated with gene flow from transgenics are no different to those from non-GM crop varieties. Certainly, there is now enough history and a plethora of examples that show the environmental damage caused by invasive species has been more devastating than the introduction of transgenic crops (Pysek 2001; Cook and Proctor 2007). Valid questions therefore have to be asked about the inconsistency between the detailed evaluations afforded transgenic varieties that data and history indicate pose no environmental risk and the lesser evaluation afforded non-GM plants.

This does of course lead into the debate about whether it should be the technique used to develop the new variety under consideration or the phenotype itself that is regulated, and furthermore places under the spotlight on the lack of international harmonisation of regulation regarding the development, commercialization and release of transgenic plants. In the real world the regulatory process can not be divorced from politics or the marketplace and as Raybould (2012) has reviewed, policies towards GMOs should clearly indicate to what extent the results of scientific research, such as that outlined in this review, will be utilised as part of the decision-making process. Any final assessment of potential risk can sometimes therefore be modified by policies which seek to place limits on the use of GM varieties in agriculture. This presents difficult decisions for regulators when the probability of gene flow is no different from the transgenic plants than from non-GM varieties of the same crop and the introduced transgene is unlikely to have any environmental impact. An example is the potential for movement of transgenes to organic or conventional fields (Bruce 2003; Jones 2006).

There are examples where transgenic plants have escaped from cultivation, including seed dispersal in canola referenced earlier in this chapter, and in creeping bentgrass (*Agrostis stolonifera*), in which gene flow by pollen and seed has been documented (Baack 2006). It is important to note that oilseed rape has not become invasive outside of cultivated and roadside habitats (Devos et al. 2012). This may reflect the fact that the potential for plants to establish outside of cultivation establishment is more a function of fitness level, not rate of gene flow from the GM plants (Chapman and Burke 2006; Burke and Riesenberg 2007). Interestingly Ellstrand et al. (1999) reviewed 13 of the world's important crops and documented that 12 of these 13 have hybridised with wild relatives at some stage somewhere in the world, suggesting that gene flow from widely grown transgenic crops, such as wheat (Brulé-Babel et al. 2006), may be inevitable. Such inevitability would indicate that risk assessment should primarily focus on the potential impacts of gene flow. It also indicates mitigation strategies are unnecessary, particularly in cases where success cannot be guaranteed (Chapman and Burke 2006). As Ellstrand (2006) has stated, thorough risk assessment may be the best form of containment.

Acknowledgments The authors would like to thank Janelle Pollock for her valuable assistance in compilation.

References

Abbot RJ, James JK, Milne RI, Giles ACM (2003) Plant introductions, hybridization and gene flow. Phil Trans R Soc Lond B 358:1123–1132
Abud S, de Souza PIM, Vianna GR, Leonardecz E, Moreira CT, Faleiro FG, Júnior JN, Monteiro PMFO, Rech EL, Aragão FJL (2007) Gene flow from transgenic to non-transgenic soybean plants in the Cerrado region of Brazil. Genet Mol Res 6:445–452
Al-Ahmad H, Galili S, Gressel J (2005) Poor competitive fitness of transgenically mitigated tobacco in competition with the wild type in a replacement series. Planta 222:372–385
Aldous DE, Offord CA, Silk JP (2011) The origin of horticulture in Australia: the early European colony in Sydney 1788–1850. Chronica Horticulture 51:8–13
Anderson NO (2007) Prevention of invasiveness in floricultural crops. In: Anderson NO (ed) Flower breeding and genetics. Springer, USA, pp 177–214
Armstrong TT, Fitzjohn RG, Newstrom LE, Wilton AD, Lee WG (2005) Transgene escape: what potential for crop–wild hybridization? Mol Ecol 14:2111–2132
Arriaga L, Huerta E, Lira-Saade R, Moreno E, Alarcon J (2006) Assessing the risk of releasing transgenic *Cucurbita* spp, in Mexico. Agric Ecosyst Environ 112:291–299
Baack EJ (2006) Engineered crops: transgenes go wild. Curr Biol 16:583–584
Bailleul D, Ollier S, Huet S, Gardarin A, Lecomte J (2012) Seed spillage from grain trailers on road verges during oilseed rape harvest: an experimental survey. Plus One 7(3):e32752. doi:10.1371/journal.pone.0032752
Baker J, Preston C (2003) Predicting the spread of herbicide resistance in Australian canola fields. Transgenic Res 12:731–737
Becker HC, Damgaad C, Karlsson D (1992) Environmental variation for outcrossing rate in rape-seed (*Brassica napus*). Theor Appl Genet 84:303–306
Beckie HJ, Warwick SI, Nair H, Seguin-Swartz G (2003) Gene flow in commercial fields of herbicide-resistant canola (*Brassica napus*). Ecol Appl 13:1276–1294
Beckie HJ, Warwick SI, Hall LM, Harker KN (2012) Pollen-mediated gene flow in wheat fields in Western Canada. AgBioforum 15:36–43
Begg GS, Cullen DW, Iannetta PPM, Squire GR (2007) Sources of uncertainty in the quantification of genetically modified oilseed rape contamination in seed lots. Transgenic Res 16:51–63
Bravo-Segretin A, Fernando, Rudoy V, Welin B, Segretin ME, Bedogni MC, Stolowicz F, Criscuolo M, Foti M, Gomez M, Lopez M, Serino G, Cabral S, Dos Santos C, Huarte M, Mentaberry A (2011) Field testing, gene flow assessment and pre-commercial studies on transgenic *Solanum tuberosum spp. tuberosum* (cv. Spunta) selected for PVY resistance in Argentina. Transgenic Res 21:967–982. doi:10.1007/s11248-011-9584-9
Bruce D (2003) Contamination, crop trials and compatibility. J Agr Environ Ethics 16:594–604
Brule´-Babel AL, Willenborg CJ, Friesen LF, van Acker RC (2006) Modeling the influence of gene flow and selection pressure on the frequency of a GE herbicide-tolerant trait in non-GE wheat and wheat volunteers. Crop Sci 46:1704–1710
Burke JM, Riesenberg LH (2007) Fitness effects of transgenic disease resistance in sunflowers. Science 300:1250
Chandler SF, Dunwell J (2008) Gene flow, risk-assessment and the environmental release of transgenic plants. Crit Rev Plant Sci 27:25–49
Chandler SF, Iván RA, Luis E, Lopez J, Claudia APO (2008) The production of transgenic carnation and rose in Colombia; a survey for wild populations and related species. In 10th International Symposium on the Biosafety of Genetically Modified Organisms (ISBGMO). Wellington, New Zealand

Chapman MA, Burke JM (2006) Letting the gene out of the bottle: the population genetics of genetically modified crops. New Phytol 170:429–443
Chun YJ, Kim DI, Park KW, Kim HJ, Jeong SC, An JH, Cho KH, Back K, Kim HM, Kim CG (2011) Gene flow from herbicide-tolerant GM rice and the heterosis of GM rice-weed F_2 progeny. Planta 807–815
Claessen D, Gilligan CA, van den Bosch F (2005) Which traits promote persistence of feral GM crops? Part 2: implications of meta-population structure. Oikos 110: 30-/42
Colbach N, Clermont-Dauphin C, Meynard JM (2001) GeneSys: a model of the influence of cropping system on gene escape from herbicide tolerant rapeseed crops to rape volunteers II. Genetic exchanges among volunteer and cropped populations in a small region. Agr Ecosys Environ 83:255–270
Cook D, Proctor W (2007) Assessing the threat of exotic plant pests. Ecol Econ 63:594–604
Cresswell JE (2000) A comparison of bumblebees' movements in uniform and aggregated distributions of their forage plant. Ecol Entomol 25:19–25
Cresswell JE (2003) Towards the theory of pollinator-mediated gene flow. Phil Trans R Soc Lond B 358:1005–1008
Cummings JL, Handley LW, MacBryde B, Tupper SK, Werner SJ, Byram ZJ (2008) Dispersal of viable row-crop seeds of commercial agriculture by farmland birds: implication for genetically modified crops. Environ Biosafety Res 7:241–252
Cureton AN, Newbury HJ, Raybould AF, Ford-Lloyd BV (2006) Genetic structure and gene flow in wild beet populations: the potential influence of habitat on transgene spread and risk assessment. J Appl Ecol 43:1203–1212
Desplanque B, Boudry P, Broomberg K, Saumitou-Laprade P, Cuguen J, Van Dijk H (1999) Genetic diversity and gene flow between wild, cultivated and weedy forms of *Beta vulgaris* L. (Chenopodiaceae), assessed by RFLP and microsatellite marker. Theor Appl Genet 98:1194–1201
Devaux C, Lavigne C, Falentin-Guyomarch H, Vautrin S, Lecomte J, Klein EK (2005) High diversity of oilseed rape pollen clouds over an agro-ecosystem indicates long-distance dispersal. Mol Ecol 14:2269–2280
Devos Y, Hails RS, Messean A, Perry JN, Squire GR (2012) Feral genetically modified herbicide tolerant oilseed rape from seed import spills: are concerns scientifically justified? Transgenic Res 21:1–21
DiFazio SP, Slavov GT, Burczyk J, Leonardi S, Strauss SH (2004) Gene flow from tree plantations and implications for transgenic risk assessment. In: Walter C, Carson M (eds) Plantation Forest Biotechnology for the 21st Century. Research signpost, Kerala, India, pp 405–422
Dlugosch KM, Whitton J (2008) Can we stop transgenes from taking a walk on the wild side? Mol Ecol 17:1167–1169
Ellstrand NC (2003) Current knowledge of gene flow in plants: implications for transgene flow. Phil Trans R Soc Lond B 358:163–1170
Ellstrand NC (2006) When crop transgenes wander in California, should we worry? Calif Agric 60:116–125
Ellstrand NC, Schierenbeck KA (2000) Hybridization as a stimulus for the evolution of invasiveness in plants? PNAS 97:7043–7050
Ellstrand NC, Prentice HC, Hancock JF (1999) Gene flow and introgression from domesticated plants into their wild relatives. Annu Rev Ecol Syst 30:539–563
Feili B, Wingartner U, Stamp P (2003) Controlling the release of pollen from genetically modified maize and increasing its grain yield by growing mixtures of male-sterile and male-fertile plants. Euphytica 130:163–165
Fenart S, Austerlitz F, Guguen J, Arnaud J (2007) Long distance pollen-mediated gene flow at a landscape level: the week beet as a case study. Mol Ecol 16:3801–3813
Ferreira JL, de Cameiro ES, L. J, Teixeira A, de Fortunata Lanes F, Cecon PR, Borem A (2007) Gene flow in common bean (*Phaseolus vulgaris L.*). Euphytica 153:165–170
FitzJohn RG, Armstrong TT, Newstrom-Lloyd LE, Wilton AD, Cochrane M (2007) Hybridisation within *Brassica* and allied genera: evaluation of potential for transgene escape. Euphytica 158:209–230

Fricke BA, Ranjan AK, Bandyopadhyay D, Becker BR (2004) Numerical simulation of genetically modified corn pollen flow. Pharm Eng 24:1–7

Funk T, Wenzel G, Schwarz G (2006) Outcrossing frequencies and distribution of transgenic oilseed rape (*Brassica napus* L.) in the nearest neighbourhood. Eur J Agron 24:26–34

Gatford KT, Basri Z, Edlington J, Lloyd J, Qureshi JA, Brettell R, Fincher GB (2006) Gene flow from transgenic wheat and barley under field conditions. Euphytica 151:383–391

Gealy DR, Bradford KJ, Hall L, Hellmich R, Raybould A, Wolt J, Zilberman D (2007) Implications of gene flow in the scale-up and commercial use of biotechnology-derived crops: Economic and policy considerations. Issue paper 37, Council for Agricultural Science and Technology, Ames, Iowa, pp 1–24

Glaser JA (2003) Transgenic plant containment. Clean Techn Environ Policy 6:3–6

Glemnitz M, Wurbs A, Roth R (2011) Derivation of regional crop sequences as an indicator for potential GMO dispersal on large spatial scales. Ecol Indic 11:964–973

Glover J (2002) Gene flow study: Implications for the release of genetically modified crops in Australia. Bureau of Rural Sciences, Canberra

Goggi AS, Lopez-Sanchez H, Caragea P, Westgate M, Arritt R, Clark CA (2007) Gene flow in maize fields with different local pollen densities. Int J Biometeorol 51:493–503

Gomez-Galera S, Twyman RM, Sparrow PAC, Van Droogenbroeck B, Custers R, Capell T, Christou P (2012) Field trials and tribulations: making sense of the regulations for experimental field trials of transgenic crops in Europe. J Plant Biotechnol 10:511–523

Gross BL, Gross HAD, Forsline PL, Richards CM, Volk GM (2012) Identification of inter-specific hybrids among domesticated apple and its wild relatives. Tree Genet Genomes. doi:10.1007/s11295-012-0509-4

Gruber S, Hüsken A, Dietz-Pfeilstetter A, Möllers C, Albrecht Weber E, Stockmann F, Thöle H, Schatzki J, Dowideit K, Renard M, Becker HC, Schiemann J, Claupein W (2012) Biological confinement strategies for seed- and pollen-mediated gene flow of GM canola (*Brassica napus* L.). AgBioforum 15:44–53

Guadagnuolo R, Savova-Bianchi D, Keller-Senften J, Felber F (2001) Search for evidence of introgression of wheat (*Triticum aestivum* L.) traits into sea barley (*Hordeum marinum* s. str. Huds.) and bearded wheatgrass (*Elymus caninus* L.) in central and northern Europe, using isozymes, RAPD and microsatellite markers. Theor Appl Genet 103:191–196

Halfhill MD, Millwood RJ, Weissinger AK, Warwick SI, Stewart CN (2003) Additive transgene expression and genetic introgression in multiple green-fluorescent protein transgenic crop x weed hybrid generations. Theor Appl Genet 107:1533–1540

Heinemann JA, El-Kawy OA (2012) Observational science in the environmental risk assessment and management of GMOs. Environ Int 45:68–71

Hoyle M, Cresswell JE (2007) The effect of wind direction on cross-pollination in wind-pollinated GM crops. Ecol Appl 17:1234–1243

Ilardi V, Barba M (2002) Assessment of functional transgene flow in tomato fields. Mol Breed 8:311–315

Jhala AJ, Bhatt H, Topinka K, Hall LM (2012) Pollen-mediated gene flow in flax (*Linum usitatissimum* L.): can genetically engineered and organic flax coexist? Heredity 106:557–566

Jones P (2006) Taking aim at a peaceful coexistence. ISB News report, Dec 2006, pp 1–11. Blacksburg : Virginia Tech, Information systems for biotechnology

Kausch AP, Hague J, Oliver M, Watrud LS, Mallory-Smith C, Meier V, Stewart CN Jr (2010) Gene flow in genetically engineered perennial grasses: lessons for modification of dedicated bioenergy crops. In: Mascia PN et al (eds) Plant biotechnology for sustainable production of energy and co-products, Biotechnology in Agriculture and Forestry 66. Springer-Verlag, Heidelberg, pp 285–297

Kiddle G, Hardinge P, Buttigieg N, Gandelman O, Pereira C, McElgunn CJ, Rizzoli M, Jackson R, Appleton N, Moore C, Tisi LC, Murray JAH (2012) GMO detection using a bioluminescent real time reporter (BART) of loop mediated isothermal amplification (LAMP) suitable for field use. BMC Biotechnol 12:15

Kobayashi K, Munemura I, Hinata K, Yamamura S (2006) Bisexual sterility conferred by the differential expression of Barnase and Barstar: a simple and efficient method of transgene containment. Plant Cell Rep 25:1347–1354

Kovinich N, Saleem A, Rintoul TL, Brown DCW, Amason JT, Miki B (2012) Coloring genetically modified soybean grains with anthocyanins by suppression of the proanthocyanidin genes *ANR1* and *ANR2*. Transgenic Res 21:757–771

Krato C, Petersen J (2012) Gene flow between imidazolinone-tolerant and: susceptible winter oilseed rape varieties. Weed Res 52:187–196

Lannenpaa M, Hassinen M, Ranki A, Holtta-Vuori MH, Lemmetyinen J, Keinonen K, Sopanen T (2005) Prevention of flower development in birch and other plants using a BpFULL1::BARNASE construct. Plant Cell Rep 24:69–78

Lavigne CE, Klein EK, Valle P, Pierre JB, Godelle B, Renard M (1998) A pollen-dispersal experiment with transgenic oilseed rape. Estimation of the average pollen dispersal of an individual plant within a field. Theor Appl Genet 96:886–896

Lee CE (2002) Evolutionary genetics of invasive species. Trends Ecol Evol 17:386–391

Lenaic P, Angevin F, Collonnier C, Messean A (2012) Impact of gene stacking on gene flow: the case of maize. Transgenic Res 21:243–256

Li Y (2012) Gene deletor: a new tool to address gene flow and food safety concerns over transgenic crop plants. Front Biol. doi:10.1007/s11515-012-1195

Lim C, Kim S, Choi Y, Park Y, Kim SU, Sung S (2007) Utilization of the *bar* gene to develop an efficient method for detection of the pollen-mediated gene flow in Chinese cabbage (*Brassica rapa spp*. *pekinensis*). Plant Biotechnol Rep 1:19–25

Liu C, Li J, Gao J, Shen Z, Lu B, Lin C (2012) A built-in mechanism to mitigate the spread of insect-resistance and herbicide-tolerance transgenes into weedy rice populations. Plus One 7(2):e31625. doi:10.1371/journal.pone.0031625

Luijten SH, de Jong TJ (2010) A baseline study of the distribution and morphology of Brassica napus L. and Brassica rapa L. in the Netherlands. Institute of Biology Leiden COGEM Report: CGM 2010–03:1–68

Luo H, Kausch AP, Hu Q, Nelson K, Wipff JK, Fricker CCR, Page OT, Moreno MA, Lee J, Hodges TK (2005) Controlling transgene escape in GM creeping bentgrass. Mol Breed 16:185–188

Lutman PJW, Berry K, Payne RW, Simpson E, Sweet JB, Champion GT, May MJ, Wightman P, Walker K, Lainsbury M (2005) Persistence of seeds from crops of conventional and herbicide tolerant oilseed rape (*Brassica napus*). Proc Roy Soc B 272:1909–1915

Mallory-Smith CA, Sanchez Olguin E (2011) Gene flow from herbicide-resistant crops: it's not just for transgenes. J Agric Food Chem 59:5813–5818

Mazzara M, Paoletti C, Crobisier P, Grazioli E, Larcher S, Berben G, De Loose M, Folch I, Henry C, Hess N, Lotte H, Janssen E, Moran G, Onori R, Van den Eede G (2012) Kernel lot distribution assessment (KeLDA): a comparative study of protein and DNA-based detection methods for GMO testing. Food Anal Methods. doi:10.1007/s12161-012-9407-5

Meagher TR, Belanger FC, Day PR (2003) Using empirical data to model transgene dispersal. Phil Trans R Soc Lond B 358:1157–1162

Messeguer J, Fogher C, Guiderdoni E, Marfa V, Catala MM, Baldi G, Mele E (2001) Field assessments of gene flow from transgenic to cultivated rice (*Oryza sativa* L.) using a herbicide resistance gene as tracer marker. Theor Appl Genet 103:1151–1159

Messeguer J, Marfa V, Catala MM, Guiderdoni E, Melei E (2004) A field study of pollen-mediated gene flow from Mediterranean GM rice to conventional rice and the red rice weed. Mol Breed 13:103–112

Nakamura N, Tems U, Fukuchi-mizutani M, Chandler SF, Matsuda Y, Takeuchi S, Matsumoto S, Tanaka Y (2011) Molecular based evidence for a lack of gene-flow between *Rosa* × *hybrida* and wild *Rosa* species in Japan. Plant Biotechnol 28:245–250

Ohmori S, Tabuchi H, Yatou O, Yoshida H (2012) Agronomic traits and gene containment capability of cleistogamous rice lines with the *superwoman 1- cleistogamy* mutation. Breed Sci 62:124–132

Orson J (2002) Gene stacking in herbicide tolerant oilseed rape: lessons from the North American experience. English Nature research report 443, Morley, United Kingdom

Osborne JL, Clarke SJ, Morris RJ, William IH, Riley JR, Smith AD, Reynolds DR, Edwards AS (1999) A landscape-scale study of bumble bee foraging range and constancy, using harmonic radar. J Appl Ecol 36:519–533

Palaudelmas M, Mele A, Monfort E, Serra J, Salvia J, Messeguer J (2012) Assessment of the influence of field size on maize gene flow using SSR analysis. Transgenic Res 21:471–483

Pascher K, Moser D, Dullinger S, Sachslehner L, Gros P, Sauberer N, Traxler A, Grabherr G, Frank T (2011) Setup, efforts and practical experiences of a monitoring program for genetically modified plants: an Austrian case study for oilseed rape and maize. Environ Sci Eur 23:12

Peter C, Meusel M, Grawe F, Katerkamp A, Cammann K, Börchers T (2001) Optical DNA-sensor chip for real-time detection of hybridization events. Fresenius J Anal Chem 371:120–127

Pla M, La Paz J, Penas G, Garcia N, Palaudelmas M, Esteve T, Messeguer J, Mele E (2006) Assessment of real-time PCR based methods for quantification. Transgenic Res 15:219–228

Pohl-Orf M, Brand U, Drießen S, Rene Hesse P, Lehnen M, Moraak C, Mucher T, Saeglitz C, von Soosten C, Bartsch D (1999) Overwintering of genetically modified sugar beet, *Beta vulgaris* L. subsp. *vulgaris*, as a source for dispersal of transgenic pollen. Euphytica 108:181–186

Pons E, Navarro A, Ollitrault P, Pen L (2011) Pollen competition as a reproductive isolation barrier represses transgene flow between compatible and co-flowering citrus genotypes. PLoS One 6(10):e25810. doi:10.1371/journal.pone.0025810

Presotto A, Ureta SM, Cantamutto M, Poverene M (2012) Effects of gene flow from IMI resistant sunflower crop to wild *Helianthus annuus* populations. Agric Ecosyst Environ 146153–161

Pysek P (2001) Past and future of predictions in plant invasions: a field test by time. Divers Distrib 7:145–151

Raybould A (2012) Can science justify regulatory decisions about the cultivation of transgenic crops? Transgenic Res 21:691–698

Raybould A, Higgins LS, Horak MJ, Layton RJ, Storer NP, De La Fuente JM, Herman RA (2012) Assessing the ecological risks from the persistence and spread of feral populations of insect-resistant transgenic maize. Transgenic Res 21:655–664

Reboud X (2003) Effect of a gap on gene flow between otherwise adjacent transgenic *Brassica napus* crops. Theor Appl Genet 106:1048–1058

Rieger MA, Lamond M, Preston C, Powles SB, Roush RT (2007) Pollen-mediated movement of herbicide resistance between commercial canola fields. Science 296:2386–2388

Rong J, Wang F, Song Z, Su J, Chen R, Lu B (2012) Scale effect on rice pollen-mediated gene flow: implications in assessing transgene flow from genetically engineered plants. Ann Appl Biol. doi:10.1111/j.1744-7348.2012.00545.x

Sandhu S, James VA, Quesenberry KH, Altpeter F (2009) Risk assessment of transgenic apomictic tetraploid bahiagrass, cytogenetics, breeding behavior and performance of intra-specific hybrids. Theor Appl Genet 119:1383–1395

Sandhu S, Blount AR, Quesenberry KH, Alpeter F (2010) Apomixis and ploidy barrier suppress pollen-mediated gene flow in field grown transgenic turf and forage grass (*Paspalum notatum* Flugge). Theor Appl Genet 121:919–929

Sanvido O, Widmer F, Winzeler M, Bigler F (2006) A framework for the design of general surveillance of genetically modified crops based on a concept for environmental post-market monitoring. J Verbr Lebensm 1(Suppl 1):5–10

Sanvido O, Aviron S, Romeis J, Bigler F (2007) Challenges and perspectives in decision-making during post-market environmental monitoring of genetically modified crops. J Verbr Lebensm 2(Suppl 1):37–40

Sanvido O, Aviron S, Bigler F (2008) Monitoring or surveillance? Balancing between theoretical frameworks and practical experiences. J Verbr Lebensm 3(Suppl 2):4–7

Sausse C, Colbach N, Young MW, Squire GR (2012) How to manage the impact of gene flow on oilseed rape grain quality? Simulation case studies of three contrasted landscapes. Eur J Agron 38:32–42

Schafer MG, Ross AA, Londo JP, Burdick CA, Lee EH, Travers SE, Van de Water PK, Sagers CL (2011) The establishment of genetically engineered canola populations in the US. PLoS One 6(10):e25736. doi:10.1371/journal.pone.0025736

Schmidt G, Schroder W (2011) Regionalisation of climate variability used for modelling the dispersal of genetically modified oil seed rape in Northern Germany. Ecol Indic 11:951–963

Scurrah M, Celis-Gamboa C, Chumbiauca S, Salas A, Visser RGF (2008) Hybridization between wild and cultivated potato species in the Peruvian Andes and biosafety implications for deployment of GM potatoes. Euphytica 164:881–892

Schulz OE, Warwick SI, Simard MJ, Legere A, Beckie HJ, Braun L, Zhu B, Mason P, Seguin-Swartz G, Stewart CN (2003) Hybridization between transgenic *Brassica napus L.* and its wild relatives: *Brassica rapa L. Raphanus raphanistrum L. Sinapis arvensis L.* and *Erucastrum gallicum* (Willd.). Theor Appl Genet 107:539

Smouse PE, Robledo-Amuncio JJ, Gonzalez-Martinez SC (2007) Implications of natural propagule flow for containment of genetically modified forest trees. Tree Genet Genome 3:141–152

Stewart CN, Halfhill MD, Warwick SI (2003) Transgene introgression from genetically modified crops to their wild relatives. Nat Genet 4:806–817

Tanaka Y, Brugliera F, Chandler S (2009) Recent progress of flower colour modification by biotechnology. Int J Mol Sci 10:5350–5369

Tardif FJ, Rajcan I, Costea M (2006) A mutation in the herbicide target site acetohydroxyacid synthase produces morphological and structural alterations and reduces fitness in *Amaranthus powellii*. New Phytol 169:251–264

Tiffney BH (2004) Vertebrate dispersal of seed plants through time. Annu Rev Ecol Evol Syst 35:1–29

Tyson RC, Wilson JB, Lane WD (2011) A mechanistic model to predict transgenic seed contamination in bee-pollinated crops validated in an apple orchard. Ecol Model 222:2084–2092

Vacher C, Kossler TM, Hochberg ME, Weis AE (2011) Impact of interspecific hybridization between crops and weedy relatives on the evolution of flowering time in weedy phenotypes. PLoS One 6(2):e14649. doi:10.1371/journal.pone.0014649

van Hengstum T, Hooftman DAP, den Nijs HCM, van Tienderen PH (2012) Does insect netting affect the containment of airborne pollen from (GM-) plants in greenhouses? Aerobiologia 28:325–335

Viljoen C, Chetty L (2011) A case study of GM maize gene flow in South Africa. Environ Sci Eur 23:8

von der Lippe M, Kowarik I (2007) Crop seed spillage along roads: a factor of uncertainty in the containment of GMO. Ecogeography 30:483–490

Walker RL, Booth EJ, Whytock GP, Walker KC (2004) Volunteer potential of genetically modified oilseed rape with altered fatty acid content. Agric Ecosyst Environ 104:653–661

Wang Z, Brummer EC (2012) Review: part of highlight on breeding strategies for forage and grass improvement. Is genetic engineering ever going to take off in forage, turf and bioenergy crop breeding? Ann Bot 110:1317–1325. doi:10.1093/aob/mcs027

Wang ZY, Lawrence R, Hopkins A, Bell J, Scott M (2004) Pollen-mediated transgene flow in the wind-pollinated grass species tall fescue (*Festuca arundinacea* Schreb.). Mol Breed 14:47–60

Wang W, Zhu T, Lai F, Fu Q (2012) Event-specific qualitative and quantitative detection of transgenic rice Kefeng-8 by characterization of the transgene flanking sequence. Eur Food Res Technol 234:477–484

Warwick SI, Legere A, Simard MJS, James T (2007) Do escaped transgenes persist in nature? The case of an herbicide resistance transgene in a weedy *Brassica rapa* population. Mol Ecol 17:1387–1395

Weekes R, Deppe C, Allnutt T, Boffey C, Morgan D, Morgan S, Bilton M, Daniels R, Henry C (2005) Crop-to-crop gene flow using farm scale sites of oilseed rape (*Brassica napus*) in the UK. Transgenic Res 14:749–759

Weekes R, Allnutt T, Boffey C, Morgan S, Bilton M, Daniels R, Henry C (2007) A study of crop-to-crop gene flow using farm scale sites of fodder maize (*Zea mays* L.) in the UK. Transgenic Res 16:203–211

Wilkinson MJ, Ford CS (2007) Estimating the potential for ecological harm from gene flow to crop wild relatives. Collect Biosafety Rev 3:42–63

Wilkinson MJ, Davenport IJ, Charters YM, Jones AE, Allainguillaume J, Butler HT, Mason DC, Raybould AF (2000) A direct regional scale estimate of transgene movement from genetically modified oilseed rape to its wild progenitors. Mol Ecol 9:983–991

Wilkinson MJ, Elliott LJ, Allainguillaume J, Shaw MW, Norris C, Welters R, Alexander M, Sweet J, Mason DC (2003) Hybridization between *Brassica napus* and *B. rapa* on a national scale in the United Kingdom. Science 302:457–459

Willenborg CJ, Brule-Babel AL, Van Acker RC (2009) Low crop plant population densities promote pollen mediated gene flow in spring wheat (*Triticum aestivum* L.). Transgenic Res 18:841–854

Wozniak CA, Martinez JC (2011) U.S. EPA regulation of plant-incorporated protectants: assessment of impacts of gene flow from pest-resistant plants. J Agric Food Chem 59:5859–5864

Yang X, Wang F, Su J, Lu BR (2012) Limited fitness advantages of crop-weed hybrid progeny containing insect-resistant transgenes (*Bt*/*CpTI*) in transgenic rice field. PLoS One 7(7):e41220. doi:10.1371/journal.pone.0041220

Zaller JG, Moser D, Drapela T, Schmoger C, Frank T (2007) Insect pests in winter oilseed rape affected by field and landscape characteristics. Basic Appl Ecol 9:682–690

Ziska LH, Gealy DR, Tomecek MB, Jackson AK, Black HL (2012) Recent and projected increases in atmospheric CO_2 concentration can enhance gene flow between wild and genetically altered rice (*Oryza sativa*). PLoS One 7(5):e37522. doi:10.1371/journal.pone.0037522

Züghart W (2010) Networks and environmental observation programs as a tool for general surveillance: first experience and future requirements. In: Breckling B, Verhoeven R (eds) Implications of GM-crop cultivation at large spatial scales, Theorie in der Ökologie 16. Peter Lang, Frankfurt, pp 24–27

Züghart W, Raps A, Wust-Saucy A, Dolezel M, Eckerstorfer M (2011) Monitoring of genetically modified organisms. Environment Agency Austria, Umweltbundesamt, REP-0305, Vienna

Chapter 11
Bioinformatics Approaches to Deciphering Alien Gene Transfer: A Comprehensive Analysis

Rajeev K. Azad, Nitish Mishra, Firoz Ahmed, and Rakesh Kaundal

Abstract A large number of bioinformatics methods have been developed in recent years for detecting gene transfers between distantly related or unrelated organisms. These have been mainly classified as parametric and phylogenetic methods. While the former methods have been frequently invoked for detecting recent gene transfers, detection of ancient gene transfers have relied upon phylogenetic methods. Numerous evidences emerging from the applications of these methods have firmly established interspecies gene transfer as a significant force-driving prokaryotic genome evolution. The focus is now shifting to assessing the extent and impact of this mechanism in eukaryotic genome evolution. The methods developed for detecting alien genes in unicellular organisms have been adapted for identifying and cataloging instances of gene transfers in multicellular organisms. A significant interest is in cataloging gene transfers in plants which have more leaky barriers to gene transfer than highly evolved animals. We review the advances in this field with a focus on alien gene transfer in plants and the bioinformatics methods frequently used to detect such transfers.

Keywords HGT quantification • Bayesian method • Bootstrapping • Phylogenetic tree • Parametric methods • UPGMA

R.K. Azad
Departments of Biological Sciences and Mathematics,
University of North Texas, Denton, TX 76203, USA

N. Mishra • F. Ahmed
The Samuel Roberts Noble Foundation, Ardmore, OK 73401, USA

R. Kaundal, Ph.D. (✉)
Department of Biochemistry & Molecular Biology, National Institute
for Microbial Forensics & Food and Agricultural Biosecurity (NIMFFAB), Oklahoma State University, 246 Noble Research Center, Stillwater, OK 74078, USA
e-mail: r.kaundal@okstate.edu

A. Pratap and J. Kumar (eds.), *Alien Gene Transfer in Crop Plants, Volume 1: Innovations, Methods and Risk Assessment*, DOI 10.1007/978-1-4614-8585-8_11,
© Springer Science+Business Media New York 2014

11.1 Introduction

Classical genetics has traditionally focused on vertical gene transfer that has helped shape the "tree thinking" in explaining the evolution of extant or extinct organisms. Advances in genome era have brought a change in this thinking, triggered by plethora of compelling evidences emerging in support of horizontal genetic inheritance, particularly in the prokaryotic domain (Ochman et al. 2000; Koonin et al. 2001; Gogarten and Townsend 2005). Horizontal Gene Transfer (HGT), also referred to as lateral gene transfer, is the transfer of genetic material between organisms by means other than parent-to-offspring (vertical) inheritance (Syvanen and Kado 1998; Ochman et al. 2000; Koonin et al. 2001; Gogarten and Townsend 2005; Keeling and Palmer 2008). While HGT is now recognized as a potent force-driving prokaryotic genome evolution, a relatively better sampling of eukaryotic genomes now available as a consequence of DNA sequencing revolution has necessitated a reassessment of the extent and impact of HGT in eukaryotic genome evolution. Numerous instances of eukaryotic HGT events reported in recent years have further galvanized this field, bringing the spotlight on gene flow among eukaryotes (Andersson 2005; Keeling and Palmer 2008; Sanchez 2011).

The evolutionary history of plant genomes is also replete with intracellular gene transfer (IGT)—the transfer of genes between organelles within a plant cell (Keeling and Palmer 2008; Bock 2010). Single to multiple instances of HGTs involving plants have been the subject of numerous recent studies and have been reviewed by several authors. Plants have served as both recipients and donors of alien genes (see Richardson and Palmer 2007; Keeling and Palmer 2008; Bock 2010 for comprehensive reviews on HGTs in plants). However, a comprehensive treatise is lacking on the methods for detecting HGTs in plants. This review is intended to provide the plant community an overview of the methods and protocols for detecting HGTs in plants. In what follows, we briefly narrate the case studies of plant HGT as reported in recent articles and reviews (Bock 2010; Keeling and Palmer 2008) and follow this up with an elaborate description of the methodology for detecting HGTs in plants.

Plants have also integrated genomes of viruses which often act as carriers for foreign DNAs. Tobacco plants have been found to have Gemini viral DNAs in their nuclear genomes (Bejarano et al. 1996). Interestingly, evidences exist even of the transfer of viral RNA sequences into plant genomes: viral sequences likely originating from closteroviruses were found in the mitochondrial genome of grape (Goremykin et al. 2009); the host's reverse transcriptase is likely to have transcribed viral RNAs to cDNAs thus facilitating their integration into the host genome.

Ralph Bock and colleagues recently designed a genetic screen to demonstrate plant to plant HGT (Stegemann and Bock 2009). Genetic engineering is a classic example of man-made HGT. Initially thought to be very rare, advances in genome sequencing and development, in parallel, of more sophisticated phylogenetic methods have helped elucidate numerous instances of natural plant–plant HGT. In most

cases, evidences appear to support cell to cell contact as a mechanism of transfer of genetic material; this has led to hypothesize that plant parasitism and natural grafting are the major factors in plant–plant HGT (Bock 2010). Plant parasites are known to be both the recipients and donors of foreign DNAs mobilized via cell to cell contact. However, the importance of other mechanisms such as transformation (uptake of naked DNA), illegitimate pollination, and vector-mediated transfers may have been understated; this needs to be reassessed in light of new genomic data emerging from plant sequencing projects.

Perhaps due to the ability of mitochondria to fuse and recombine, mitochondrion–mitochondrion HGTs are much more prevalent than plastid-initiated transfers. Unlike chloroplasts, plant mitochondria contain active DNA uptake system (Koulintchenko et al. 2003; Logan 2006). Of the few cases of alien gene transfer involving chloroplast genomes, it has been argued that these transfers may actually be mediated by mitochondria and less likely be de novo chloroplast HGTs. A more plausible explanation for the presence of chloroplast *pvs-trnA* genic sequence in the mitochondrial genome of *Phaseolus* is the IGT of this sequence from donor's chloroplast to its mitochondrion followed by mitochondrion to mitochondrion HGT (Woloszynska et al. 2004). The transfer of whole chloroplast genome by performing grafting experiments involving *Nicotiana tabacum* (donor), *Nicotiana glauca* (recipient), and *Nicotiana benthamiana* (recipient) has been demonstrated recently (Stegemann et al. 2012). This study thus provides a strong case for natural grafting as a possible mechanism for chloroplast transfer among plant species.

Nuclear genes are also not immune to plant–plant HGT. HGT of a transposon, MULE (Mu-like elements), involving nuclear genomes of *Setaria* and *Oryza*, could be an example of vector-mediated nuclear HGT (Diao et al. 2006). A just published study on the evolution of C_4 photosynthesis trait in the grass lineage *Alloteropsis* implicates plant–plant nuclear HGT involving donors from the C_4 lineage that diverged from *Alloteropsis* more than 20 million years ago (Christin et al. 2012).

The horizontal acquisition of alien DNAs is not restricted to a single gene or multiple genes but may even involve fragments of genes. A few cases of horizontal intron transfer in plants have been reported: *Peperomia polybotrya*, a basal angiosperm, has integrated an intron from a fungal donor into its mitochondrial *cox1* gene (Vaughn et al. 1995); another example is a self-splicing intron likely originating from a cyanobacterium found in the *psbA* gene of the alga *Euglena myxocylindracea* (Sheveleva and Hallick 2004). Won and Renner provided an striking example of plant to plant horizontal intron transfer: the intron 2 belonging to group II introns along with its flanking exons from the mitochondrial gene *nad1*of an asteroid (angiosperm) was transferred to Gnetum (gymnosperms) 2–5 million year ago (Won and Renner 2003). An interesting case is of the *rps11* gene in the mitochondrial genome of *Sanguinaria*, an eudicot; this gene has a chimeric structure with its 3′ half acquired from a monocot (Bergthorsson et al. 2003; Richardson and Palmer 2007).

11.2 Mechanisms of HGT

Although cell to cell contact has been the most cited mechanism of gene transfer in plants, the contributions of other mechanisms including transformation and transduction might have remained underestimated. Plants can acquire alien DNAs via all the three basic mechanisms reported for gene transfer among prokaryotes (Ochman et al. 2000).

11.2.1 Transformation

Through this mechanism, a recipient cell can take in naked DNA directly from the environment. Although a common mechanism for gene transfer among bacteria, this is less common among eukaryotes. Short DNA fragments can be readily transferred using this mechanism.

11.2.2 Conjugation

Conjugation requires the physical contact of donor and recipient cells and the transfer is mediated through plasmids. This process can facilitate transfer of genetic material between distantly related organisms, and by its very nature, conjugation can move large fragments of DNAs.

11.2.3 Transduction

In transduction, the transfer of genetic material is mediated through bacteriophages which package alien DNAs from a donor cell and inject it into a recipient cell during infection. The amount of transferred DNAs is limited by the size of phage.

However, there are several barriers to HGT, which help to protect the recipient organism from deleterious effects by maintaining the integrity of the host genome (Kurland et al. 2003; Kurland 2005; Thomas and Nielsen 2005). These barriers include physiological state of donor and recipient cell, adaptability of the incoming DNA into a recipient cell, surface exclusion for the plasmid-mediated transfers, cleavage of foreign DNA by recipient's restriction system, hindrance to plasmid replication within recipient cell, successful integration into host genome, and the likelihood of acquired gene's expression within the recipient system. An understanding of these barriers will help advance the field of genetic engineering, the artificial counterpart of natural HGT, which has become an important tool to secure a desired phenotype by augmenting the physiological repertoire of an organism through gene transfer.

11.3 Quantifying HGT

The prevalence and significance of HGT has necessitated the development of novel methodologies for robust quantification of horizontal gene flow. Detection of HGT is often confounded by many factors and no single method is capable of addressing this problem. Therefore, several complementary approaches have been proposed, and a combination of disparate approaches appears to address the detection of HGT more convincingly (Azad and Lawrence 2012). The extent and impact of HGT in plants have not been realized until recently, mainly due to lack of sequenced genomes of close relatives of a species of interest, and also because of the limitation of experimental methods frequently invoked by plant biologists in cataloging gene transfer events. Post genome sequencing revolution, detection of alien genes has come to rely upon computational methods which can assess, on a genome-wide scale, the extent and consequence of HGT in plant evolution. Several computational methods have been developed to detect horizontally transferred genes, which can be categorized into two types: phylogenetic methods and parametric methods (sometimes also called composition based or surrogate methods) (Azad and Lawrence 2012). While the former methods have almost always been invoked in detecting alien genes in plants, the latter methods have not yet been seriously explored for assessing gene transfer among eukaryotes. We discuss below the principles underlying both approaches, and the different questions or hypothesis they test to infer alien genes in a given genome.

11.4 Phylogenetic Methods for Alien Gene Detection

This class of methods is focused on detecting aberrant phylogenetic patterns, that is, the gene relationships that differ significantly from the canonical organismal phylogeny (Beiko and Hamilton 2006; Poptsova 2009). Phylogenetic methods, as the name suggests, infer relationships by constructing phylogenetic trees based on complex morphological features or nucleotide sequences of genes. This is perhaps the most commonly used approach for detecting HGT in eukaryotes including plants (Keeling and Palmer 2008). HGT is primarily inferred by detecting discrepancies in the phylogenetic tree of orthologous genes when compared to species tree which represents the overall phylogenetic relationships among all considered species. The requirement of presence of homologues of a gene in *all* genomes of interest limits the applicability of phylogenetic tree-based methods; complementary phylogenetic methods that do not explicitly require building trees in order to infer alien genes have also been developed. We summarize below the frequently invoked phylogenetic approaches for alien gene detection.

11.4.1 *Phylogenetic Tree*

To construct a phylogenetic tree representing relationships among organisms, highly conserved molecular sequences of DNA, RNA, or protein molecules that have evolved slowly yet engendered subtle differences to reliably compare taxa over large evolutionary distances have been used. A frequently used phylogenetic marker, initially proposed by Woese and colleagues, is the nucleotide sequence of 16S small subunit ribosomal RNA gene which has primarily been relied upon for inferring organismal phylogeny (Woese et al. 1990; Woese 1991; Olsen and Woese 1993). However, organismal phylogenies inferred from other conserved sequences differ among themselves and from ribosomal RNA phylogeny (Hilario and Gogarten 1993; Brown et al. 1994; Gogarten 1995; Nesbo et al. 2001; Poptsova 2009). This has led to developing other strategies for extracting a reliable species or organismal tree from molecular sequence data. One approach is to find a consensus from orthologous gene trees. Variants of consensus methods include strict consensus, majority-rule consensus (Day and McMorris 1992; Dong et al. 2010), Adams consensus (Adams 1972), and super tree consensus methods (Bininda-Emonds and Sanderson 2001; Eulenstein et al. 2004; Bininda-Emonds 2005; Nguyen et al. 2012; Swenson et al. 2012). This is based on the premise that a majority of genes are acquired vertically and therefore the phylogenetic signal representing vertical inheritance can be reconciled to an acceptable degree of confidence from the orthologous gene trees. Another approach to infer species tree is based on concatenation of orthologous gene alignments (Wolf et al. 2002), referred to as super matrix approach (Lapierre et al. 2012). Both super tree and super matrix methods are used frequently. A recent study used genome simulations to assess the accuracy of these methods in recovering species tree when subjected to HGT (Lapierre et al. 2012). The methods were found sensitive to the amount of HGT. The super matrix approach performed better for low amount of HGT, while the super tree approach was more accurate for moderate amount of HGT. Any prior information on the frequencies of HGT in the evolution of organisms of interest could thus help in selecting the most appropriate method. The species tree thus obtained represents the null hypothesis that there was no HGT in the history of orthologous genes. If a gene tree deviates significantly from species tree, this indicates an HGT in the history of this gene. One major advantage of this approach is that the likely scenarios of horizontal gene flow are assessed directly and the direction of gene flow determined unambiguously, thus identifying the recipient and donor organisms involved in gene transfer. Because of this attribute, phylogenetic tree methods have been often invoked to infer roadmap of gene transfers. There are five steps to phylogenetic tree construction:

11.4.1.1 Identify Orthologues of a Gene of Interest

Identification of homologues of a gene diverging following speciation events, namely, the orthologues, is the first step in phylogenetic gene tree construction.

Given a set of genes, one can use all against all BLAST similarity search (Altschul et al. 1990) to identify reciprocal best hits within the set followed by elimination of paralogous genes (homologous as a consequence of gene duplication). There are databases of orthologous genes that one can also use such as Clusters of Orthologous Groups (COGs) (Tatusov et al. 2000), OrthoMCL-DB (Chen et al. 2006), and MultiParanoid (Alexeyenko et al. 2006). NCBI's HomoloGene is a useful repository (http://www.ncbi.nlm.nih.gov/homologene) for eukaryotic orthologues and paralogues.

11.4.1.2 Perform a Multiple Sequence Alignment of Gene Orthologues

Dynamic programming methods as well as heuristic methods have been developed for multiple sequence alignment of members of gene or protein families. Programs based on progressive alignment methods such as ClustalW (Thompson et al. 2002) and MUSCLE (Edgar 2004) use a guide tree to perform multiple sequence alignment, progressively assembling most similar pair of sequences into a multiple alignment. Iterative refinement methods refine the progressive alignment by recursively aligning a sequence to the rest of the sequences in the progressive alignment. This is repeated for each sequence in the alignment or until the convergence of the alignment score (Durbin et al. 1998). Popular programs implementing iterative refinement include (Katoh et al. 2009), INTERALIGN (Pible et al. 2005) and PRALINE (Simossis and Heringa 2003, 2005). Probabilistic models, namely, the profile hidden Markov models, have been used in the consistency-based methods to achieve greater accuracy in alignment (e.g., the ProbCons program) (Do et al. 2005).

11.4.1.3 Select an Evolutionary Model of Nucleotide/Amino Acid Substitution

A simple approach to measure differences between two sequences in an alignment is to count the alignment positions where the residues (nucleotides or amino acids) differ and divide this difference by the alignment length. More sophisticated substitution models include the Jukes-Cantor model and Kimura 2- or 3-parameter model (Durbin et al. 1998).

11.4.1.4 Use One of the Tree Construction Methods

The five tree construction methods are classified as distance-based methods (UPGMA and neighbor-joining), character-based methods (maximum parsimony), and model-based methods (maximum likelihood and Bayesian) (Durbin et al. 1998; Pevsner 2003). Distance-based methods use a distance measure to perform pairwise comparison of DNA or protein sequences. This way two sequences with least nucleotide or amino acid changes observed in their alignment form the first two

sister branches of the phylogenetic tree, joining at a node representing their common ancestor. This process is repeated recursively to generate other branches and ancestral nodes of the tree. In contrast, character-based methods process the information within multiple sequence alignment all at once; maximum parsimony approach accomplishes this by evaluating the likely scenarios in evolution giving rise to variations in characters (nucleotides or amino acids) at the informative sites of multiple sequence alignment. The tree postulating relationship among given taxa with minimal number of character variations or mutations is the most parsimonious explanation of relationship among taxa and is therefore considered the optimal tree given the sequence data. The maximum likelihood methods are based on the premise that the most likely tree representing the given data is the one that maximizes the likelihood of generating the observed data. Here, all possible trees with different topologies and branch lengths are explored in order to find the optimal tree representing the evolutionary history of the given sequence data. Unlike maximum parsimony which requires counting of nucleotide or amino acid substitutions, maximum likelihood associates probability to each evolutionary event and so requires specifying probabilistic evolutionary models. Bayesian methods are similar in spirit to the maximum likelihood methods, searching for most probable tree given the data; however, the optimal tree is now inferred from the posterior distribution of trees computed via Markov Chain Monte Carlo (MCMC) (Gelman and Rubin 1996) simulations. Bayesian methods add the flexibility to incorporate prior information about the model (tree parameters, etc.). The above approaches have been implemented in different software programs such as PHYLIP (distance based, maximum parsimony, maximum likelihood) (Felsenstein 1989), PAUP (maximum parsimony) (Swofford 1998), TREE-PUZZLE (maximum likelihood) (Schmidt et al. 2002; Schmidt and von Haeseler 2007), and MrBayes (Bayesian) (Huelsenbeck and Ronquist 2001).

11.4.1.5 Evaluate Trees Using Bootstrapping

Bootstrapping methods are used to assess confidence over the branching patterns of a tree topology (Efron et al. 1996; Durbin et al. 1998). Each node with bifurcating or multi-furcating branches is given a confidence score as follows. Columns from a multiple sequence alignment are selected randomly and with replacement in order to construct a random replicate of the original alignment. Confidence on a clade in a tree is obtained as the proportion of times that clade appears in the random replicates of the tree.

The next step in this sequence of protocols is to assess the gene tree against the background (organismal) tree. Likelihood-based methods such as Shimodaira-Hasegawa (S-H) (Shimodaira and Hasegawa 1999), Kishino-Hasegawa (K-H) (Kishino and Hasegawa 1989), and Approximately Unbiased (AU) tests (Shimodaira 2002) are frequently used for this purpose. These tests allow testing the null hypothesis that a gene tree is similar to the organismal tree; if the p-value for the likelihood statistics is less than a significance threshold (typically 0.05 or less), the null

hypothesis is rejected thus inferring HGT in the evolutionary history of the gene. The other approach is to compute Robinson-Foulds (R-F) distance (Robinson and Foulds 1981) between gene tree and species tree, which is essentially the minimum number of operations required to transform a gene tree into a species tree. Assuming most genes in an organism to have been vertically inherited, a significant deviation from the mean of R-F distances between gene trees and species tree is an indicator of HGT. Similar in spirit to R-F distance is the sub-tree prune-and-graft (SPR) distance (Swofford and Olsen 1990), which is equivalent to minimum number of rearrangements required to change the topology of a gene tree to that of the species tree.

A nontrivial issue in alien gene detection is the fidelity of the phylogenetic methods. To assess the phylogenetic methods, one must have orthologous gene sets with no history of HGT (the "null" datasets for estimating the false-positive rate) and orthologous gene sets with history of HGT (for estimating false-negative rate). Since evolutionary events are often difficult to validate, alternative approaches have been developed to construct test datasets. One approach is based on absolute consensus; if none of the phylogenetic methods find a support for HGT in the history of orthologous genes, the set of such genes defines the "backbone" signifying vertical inheritance. Gene transfers could be simulated within the same dataset to construct a set of genes with one or more HGT events happening in the course of their evolution. The power of a phylogenetic method could thus be assessed on these datasets. The other approach is to simulate species evolution. Evolsimulator (Beiko and Charlebois 2007) starts with a set of genes in an ancestral genome which is evolved through speciation and other evolutionary processes sans the HGT. This gives sets of orthologous genes that have evolved vertically and therefore could be used for estimating the false-positive rate. One can also simulate HGT in the history of orthologous genes and this data could be used for estimating false-negative rate.

Keeling and Palmer (2008) elucidated six likely scenarios of gene transfer which include (1) duplicative transfer, where the recipient genome retains both the horizontally acquired and original copies of a homologous gene, (2) recent homologous replacement where a gene transfer event between extant organisms results in replacement of the recipient's gene by a homologous copy from a distantly related donor, (3) ancient homologous replacement where the homologous gene replacement involves ancestors of different lineages, (4) duplicative transfer with differential loss where the lineage-specific gene losses follow the gene transfer event, (5) sequential transfer where the same gene gets transferred more than once to different lineages, and (6) new gene transfer where a gene of recent origin in a lineage gets transferred to another lineage with no history of this gene via illegitimate recombination (Fig. 11.1). Phylogenetic methods are thus subjected to different sets of challenges arising from different scenarios of gene transfer, and the differential gene loss, in particular, has a deeper confounding effect on deciphering HGT.

In addition to lineage-specific gene loss, other confounding factors in HGT detection via tree building include biased mutation rates, improper clade selection, long branch length attraction, and segregation of paralogues (Kurland et al. 2003; Kurland 2005). Further, the phylogenetic HGT prediction is only as good as the consensus organismal tree which is hard to reconcile despite recent advances.

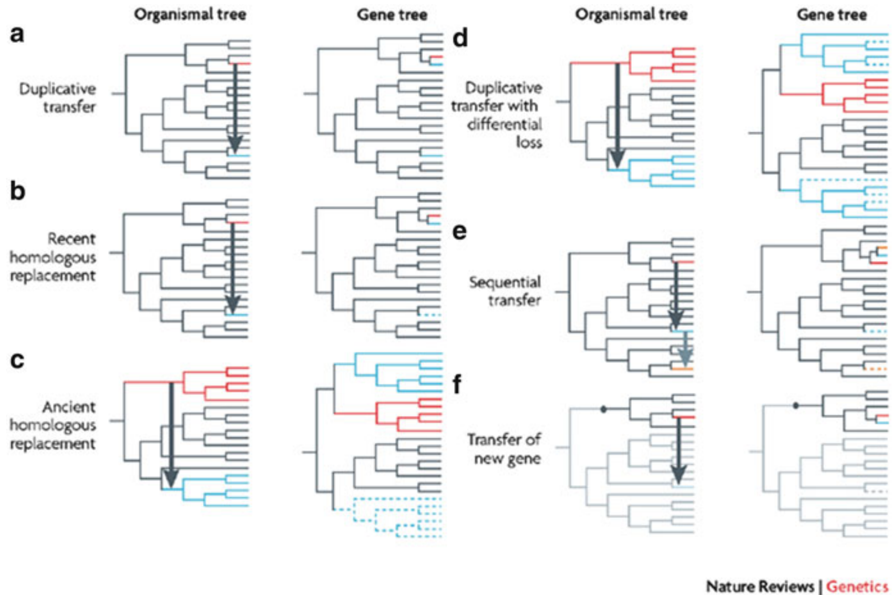

Fig. 11.1 Incongruent gene phylogenies as a consequence of different kinds of gene transfers (Keeling and Palmer 2008; Reprinted by permission from Nature Publishing Group: Nature Reviews Genetics)

Perhaps one of the biggest bottlenecks is determining the phylogeny of "orphan" genes (those lacking homologues in the database). It is plausible that many orphan genes might have arrived horizontally; however, due to the absence of their orthologues, phylogenetic methods cannot be applied to detect orphan gene transfer. Despite these shortcomings, phylogenetic methods are considered most reliable in inferring ancient gene transfer.

11.4.2 Unusual Phyletic Pattern

Phylogenetic tree methods may lead to confounding interpretation as discussed above. At the same time, comparative genomics provide alternative routes to avoid the vagaries of the tree methods. This approach examines the genomes of closely related organisms for the presence of unusual phyletic pattern (Lander et al. 2001; Gophna et al. 2006; Vernikos and Parkhill 2008; Arvey et al. 2009). If a gene is present in the genome of an organism but absent in the genomes of closely related organisms, it is likely to have been acquired horizontally. This approach is now receiving greater acceptance due to more reliable sampling of closely related genomes.

However, this approach is also not free from caveats. The unusual phyletic pattern may be a consequence of lineage-specific gene loss than a gene gain. Further, the gene of interest may actually be a paralogue that has diverged following the duplication event and therefore does not appear to reside in the closely related genomes. Incomplete genome or the loss of original gene copy complicates the verification of this hypothesis. Another caveat is that the gene displaying unusual pattern might be evolving rapidly, due to selective pressure resulting in unusually high substitution rate. This could occur either in the gene of interest or in the orthologues of this gene in the related genomes. Frequent gene or genome rearrangement and the requirement of multiple strains of closely related species potentially limit the applicability of this approach to lineages or clades with good sampling of completely sequenced genomes. Further, the arbitrary choice of phylogenetic distance to define close or distant relationship renders this approach susceptible to incorrect interpretations.

11.4.3 Similar Genes in Distant Lineages

Pairwise sequence similarity methods such as BLAST are used to find genes with unusually high degree of similarity in otherwise distant lineages (Aravind et al. 1998; Nelson et al. 1999; Lander et al. 2001; Armbrust et al. 2004). Many interdomain gene transfers were reported through this approach. For example, if a gene in a plant appears more similar to bacterial genes than plant genes, this presents an evidence of transfer of this gene from a bacterial genome to a plant genome. Note that such transfers are easier to detect due to large evolutionary distance between donor and recipient organisms, and thus much stronger phylogenetic signal to resolve in order to infer gene transfer events. Although relatively rare, interdomain transfers have contributed significantly to shaping the evolution of extant organisms. Such transfer events have been documented as important players in evolutionary and ecological processes such as host-parasite interaction. However, these methods are also not immune to the vagaries of the comparative approaches. Perhaps, they are highly vulnerable to misinterpretation, and a well-known example comes from the human genome project which reported hundreds of bacterial genes in human genome (Lander et al. 2001) but was strongly refuted by subsequent studies (Salzberg et al. 2001; Stanhope et al. 2001). Therefore, one needs to carefully weigh the caveats including high conservation of the gene of interest coupled with the likely scenario of its differential loss in certain lineages, and also the feasibility of convergent evolution contributing this non-concordance before inferring HGT using this approach. Despite its inherent limitations, this approach is still used frequently but in conjunction with other approaches to add confidence over predictions (Richards et al. 2009).

11.4.4 *Phylogenetic Methods and Their Inferences: Most Comprehensive yet Most Confounding*

The scope and advantages of phylogenetic methods arise from their inherent ability to construct a roadmap of gene transfer, identifying both the recipient and donor organisms as well as the paths of gene flow. However, the just discussed other likely scenarios that may also explain the observed pattern could be sometimes overwhelming and it is often nontrivial to assign probabilities to each of the alternative scenarios let alone unambiguously rule out the rest in favor of one. By the very nature of their design, the success of these methods solely depends on the breadth and depth of sequence database. Given the sheer complexity and sophistications involved in tree making, quantifying horizontal gene flow at genome scale could be difficult. Although alternative approaches have been developed to make this task computationally less intensive, for example, by prioritizing genes that are more likely to have evolved via HGT, but this issue still remains at the core of phylogenetic limitations (Beiko and Hamilton 2006; Beiko and Ragan 2008, 2009).

In order to catalog plant–fungi HGT, Richards et al. (2009) recently proposed a pipeline that excluded a large proportion of plant genes from further downstream phylogenetic tree analysis; only those plant genes showing the greatest similarity to fungal genes (excluding other plant genes) were selected for phylogenetic tree analysis. Although HGT quantification becomes much faster and applicable genome-wide, this approach is biased towards detecting recent gene transfers from distinct lineages. Another attempt to address this problem culminated in the development of Efficient Evaluation of Edit Paths (EEEP) method (Beiko and Hamilton 2006), however, the computer memory still remained a limiting factor, and further it is hard to resolve the equally parsimonious edit paths and the direction of gene transfer.

Consensus-based methods have been developed to infer an organismal tree, however, since the HGT prediction methods are highly sensitive to heuristically derived organismal tree, any error in extracting the consensus phylogenetic signal would have a profound negative effect on the reliability of inference on all genes being tested for HGT hypothesis.

11.5 Parametric Methods for Alien Gene Detection

This class of methods is based on the premise that an alien gene having evolved in a different (donor) genomic context appears compositionally distinct in the recipient genome context, and could therefore be identified by measuring the compositional disparities against the recipient genome background. Note that ancient transfers are difficult to detect using parametric methods as these alien genes, constrained by recipient's mutation-selection pressure, may have their composition ameliorated to that of the recipient genome (Lawrence and Ochman 1997). However, since most acquired genes are lost over the course of evolution, the repertoire of

alien genes in a genome is replete with recently acquired genes. And, therefore, parametric methods have often been invoked to assess the scale and impact of recent gene transfers, particularly, among the microbes (Lawrence and Ochman 1998; Ochman et al. 2000). These methods have sparingly been used for detecting HGT in plants, partly because most remarkable developments in parametric alien gene detection have happened only recently (Arvey et al. 2009; Azad and Lawrence 2011, 2012). The earlier parametric methods used simpler discrimination criteria such as G+C compositional bias to identify alien genes (Lawrence and Ochman 1998); more recent parametric methods have much greater sophistication and have shown consistently high performance in detecting bacterial gene transfer (Vernikos and Parkhill 2006; Azad and Lawrence 2007, 2011; Arvey et al. 2009; Azad and Li 2013). Many of these recent methods hold the promise to robustly quantify the horizontal gene flow among eukaryotes. Since these methods are computationally less intensive and amenable to genome scale analysis, their adaptation for detecting gene transfers in plants will significantly advance our understanding of plant evolution via HGT.

11.5.1 Bottom Up Parametric Methods

These methods perform gene-by-gene analysis to classify each gene as either native or alien (see, for example, Lawrence and Ochman 1998; Garcia-Vallve et al. 2000). Alternatively, without gene information, one can move a fixed size window along a genome sequence and assess the compositional character of the region within the window (Karlin 1998). The bottom up methods can be further categorized as clustering and non-clustering methods.

11.5.1.1 Gene Clustering Methods

The fundamental principle underlying gene clustering methods is that the genes that have evolved under similar evolutionary constraints appear similar to each other and thus could be grouped together and discriminated against other groups having similar genes. Since majority of the genes in a genome are ancestral or native genes, the largest cluster of genes correspond to the genome backbone and all other smaller clusters harbor similar genes that are likely arising from different donor sources. A popular approach to group similar genes is to first randomly assort given genes into k number of clusters, and then compute the cluster center (represents the mean of the sequence properties, e.g., nucleotide frequencies, in a cluster) of each cluster, followed by reassignment of genes to the clusters with closest cluster center. This process is repeated until convergence, that is, further reassignment will result in the same cluster configuration. Variants of k-means clustering procedure were used for grouping genes with similar compositional pattern in earlier studies (Médigue et al. 1991; Hayes and Borodovsky 1998). One serious limitation of this approach is that

one has to specify a priori the number of clusters (value of k) which is often unknown for the given data. For identifying alien genes, a naïvely chosen value of k (e.g., $k=2$) may result in high misclassification errors (Azad and Lawrence 2005). To address this problem, Azad and Lawrence (2007) developed a gene clustering method that identifies the number of clusters inherent to genome heterogeneity in a hypothesis testing framework. Beginning with single gene clusters, a hierarchical agglomerative clustering procedure allows to group recursively two most similar gene clusters. This recursion is halted when the difference between gene clusters in any cluster pair becomes significantly large. The largest cluster is identified as native and the remaining smaller clusters as alien. While this procedure reduced the misclassification errors significantly in comparison to other methods, combining it with biological information such as gene context information for reassigning the compositionally ambiguous genes further reduced the misclassification errors (Azad and Lawrence 2007).

11.5.1.2 Non-clustering Methods

Since a large majority of genes in a typical genome are ancestral, the genome composition (average over all genes) is often taken to represent the composition of ancestral genes. One can thus infer alien genes by assessing the compositional atypicality of a gene against the genome background. Most parametric methods are based on this premise yet they test different hypothesis and thus often lead to non-convergent predictions (Ragan 2001; Lawrence and Ochman 2002). The most simple, and perhaps most used, among these methods, is to measure the discrepancies in nucleotide composition of a gene vis-à-vis the whole genome. Lawrence and Ochman (1998) proposed that if the G+C composition at first and third codon position of a gene deviates significantly from the respective means for all genes, the gene in question is likely an alien gene. Karlin (1998) went a step further, suggesting that the dinucleotide compositional bias is a stronger indicator of atypicality, perhaps inspired by the dinucleotide compositional differences he observed in pairwise comparison of genomes of different species, which led him to propose that dinucleotide composition represents genomic signature, and thus could be exploited to detect alien genes which exemplify genomic signatures of donor organisms and so appear distinct from recipient organism's genomic signature. More recent studies suggest that higher order k-mers carry greater discriminative power and thus can potentially improve alien detection (Tsirigos and Rigoutsos 2005a). Design-Island (Chatterjee et al. 2008) and a chaos game representation-based method (Deschavanne et al. 1999; Dufraigne et al. 2005) were developed for exploiting the power of tetra-nucleotide compositional bias in alien gene detection. Advantages of higher order k-mers include the utilization of codon usage information lying within trimers or longer oligomers ($k>3$), and better predictive abilities encoded within nucleotide ordering patterns arising as a consequence of differential evolutionary forces acting upon genomes of different organisms. Nakamura et al. (2004) used

hexamer frequency as a discriminant criterion in a Bayesian formalism, Horizontal Transfer Index, to catalog alien genes in bacterial genomes. Another Bayesian approach, the naïve Bayesian classifier, also used oligomer frequencies to compute the a posteriori probability of a genomic segment to be originating from one of the possible donor sources (Sandberg et al. 2001). However, there is a caveat to the usage of higher order *k*-mers: longer oligomers carry greater predictive ability only if there is a good sampling (recurrence) of longer oligomers in the data. For example, a hexamer, which does not occur frequently enough in the data, cannot be used to predict the nucleotide that just succeeds this hexamer in a DNA sequence. This issue could be circumvented to an extent by using a variable length *k*-mer model, also called interpolated Markov model (Salzberg et al. 1998; Azad and Borodovsky 2004), which was implemented in the IVOM *a.k.a.* Alien Hunter program (Vernikos and Parkhill 2006). Another critical aspect of this class of methods is the choice of measure or model framework for assessing the compositional difference between DNA sequences of interest. Arvey et al. (2009) have shown that an entropy-based measure outperforms a covariance-based measure (Tsirigos and Rigoutsos 2005a) even when the former uses just the nucleotide composition while the latter uses its "optimal" octanucleotide composition as the discriminate criterion. The octanucleotide compositional bias was also exploited in a Support Vector Machine framework (Tsirigos and Rigoutsos 2005b), a frequently invoked supervised learning procedure used successfully in solving a range of biological problems, e.g., disease forecasting (Kaundal et al. 2006), subcellular localization prediction (Kaundal and Raghava 2009; Kaundal et al. 2010). Though the octanucleotide composition approach was outperformed by other methods that used dinucleotide composition in a model selection framework or codon usage in a hypothesis testing framework (Azad and Lawrence 2007). Note that where the gene information is available, one can use codon usage information to exploit the atypical codon usage biases of alien genes. This was implemented in methods by Karlin (1998), and Azad and Lawrence (2007).

11.5.2 Top Down Parametric Methods

While bottom up parametric methods robustly classify the strongly typical and atypical genes, *all* bottom up methods have difficulty in classifying compositionally ambiguous genes. Given that genes often arrive *en masse*, with tens to hundreds acquired in a single transfer event, misclassification of compositionally ambiguous genes in these alien gene islands (also, genomic islands) will lead to overestimation of gene transfer events. Consequently, it will lead to a fragmented structure of otherwise large genome islands. To address this problem, Azad and Lawrence (2011) have recently suggested the use of gene context and operon structural information embedded within the genome of an organism to classify compositionally ambiguous genes in a multiple threshold model framework. However, a robust identification of large acquired regions with dozens of alien genes requires a different approach that

separate in this particular case can not just simultaneously analyze multiple genes within an acquired region but be able to do so without regard to gene information and thus predict island boundaries more precisely, which can even lie in non-genic regions. Arvey et al. (2009) have shown that this can be realized in a top down framework. They used a recursive segmentation procedure to divide a given genome sequence recursively into compositionally homogeneous regions within a hypothesis framework. If a homogeneous segment thus obtained was found sufficiently atypical vis-à-vis the genome composition, it was labeled alien. As a consequence, all genes—whether strongly, moderately, or weakly atypical—harbored by this segment, were labeled alien. This class of methods, having demonstrated their power in delineating genomic islands in bacterial genomes, holds a great promise in deciphering large acquired regions in eukaryotic genomes, including genomic islands in plant genomes, where often the gene annotation is incomplete or unavailable.

11.5.3 Parametric Methods and Their Inferences

Parametric methods are becoming increasingly popular because of their simplicity, genome-wide applicability, interpretability, and ease in their implementation. One of the biggest advantages of this class of methods is that these methods do not require multiple related (or sometimes, unrelated) genomes to infer alien genes. The sole input is the genome of an organism (either the whole genome sequence or the sequences of all genes). Alien genes are identified without regard to the presence or absence of their homologues in the genomes of other organisms. However, these methods often generate non-convergent results, which is perhaps because of their testing different hypotheses for being alien (Lawrence and Ochman 2002). Azad and Lawrence (2005) have argued that this is rather a strength than a weakness, for this offers an opportunity to combine the complementary strengths of different parametric methods. To buttress this claim, they combined the predictions from two methods, one using dinucleotide composition and the other using codon usage bias as discriminant criterion, and showed that a simple union of predictions at conservative thresholds significantly minimizes both Type I and Type II errors of misclassification (Azad and Lawrence 2005). Though both, bottom up and top down parametric methods, were designed for different purposes, integration of the two disparate methods will augment the power in delineating and characterizing the compositionally aberrant regions (Arvey et al. 2009; Azad and Lawrence 2012).

Like phylogenetic methods, which suffer from the vagaries related to consensus phylogenetic signals, the performance of bottom up parametric methods is also a function of consensus signal. Often the whole genome composition is assumed to represent the "native" parametric signal; however, this assumption would be severely violated for genomes that have undergone rampant gene transfers. In contrast, bottom up hierarchical clustering methods do not suffer from this limitation. Other caveats include the failure to detect HGT among phylogenetically similar organisms (for example, transfer between *E. coli* and *S. enterica*) and false predictions of otherwise differentially evolving native genes.

11.6 Conclusions

A survey of the recent developments in quantifying HGT in plants highlights the importance of gene transfer in plant genome evolution. It was not long ago that HGT was perceived extremely rare in higher eukaryotes (unlike microbes that swap genetic material frequently among themselves), but this long-held perception has now come into question due to emergence of numerous evidences supporting HGT in eukaryotes, and particularly bolstered by a plethora of plant HGTs reported in recent years. This has infused renewed interest and enthusiasm in the field. There are bottlenecks that must be addressed; this includes the tendency to filter bacterial DNAs if any during eukaryotic genome assembly, and more importantly, conflicting predictions generated by different methods. Integrative approaches to reconcile conflicting signals have remained elusive despite forceful arguments put forward in support of this (Arvey et al. 2009). Parametric methods have come a long way, and with the inclusion of more sophisticated, top down methods in parametric repertoire (Arvey et al. 2009), time is just ripe to exploit the power of these methods, which has, rather surprisingly, been overlooked for alien gene detection in plants. Future strategies should focus on integration of phylogenetic and parametric methods for robustly cataloging both ancient and recent gene transfers in the evolutionary history of plants.

Acknowledgement This work is supported by faculty start-up funds to R.K. from NIMFFAB, Department of Biochemistry & Molecular Biology, Oklahoma State University and similar start-up funds from the University of North Texas to R.K.A. The authors thank the anonymous referees for critically reviewing and help in improving the book chapter.

References

Adams EN III (1972) Consensus techniques and the comparison of taxonomic trees. Syst Biol 21:390–397

Alexeyenko A, Tamas I, Liu G, Sonnhammer EL (2006) Automatic clustering of orthologs and inparalogs shared by multiple proteomes. Bioinformatics 22:e9–e15

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Andersson JO (2005) Lateral gene transfer in eukaryotes. Cell Mol Life Sci 62:1182–1197

Aravind L, Tatusov RL, Wolf YI, Walker DR, Koonin EV (1998) Evidence for massive gene exchange between archaeal and bacterial hyperthermophiles. Trends Genet 14:442–444

Armbrust EV, Berges JA, Bowler C, Green BR, Martinez D, Putnam NH et al (2004) The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. Science 306:79–86

Arvey AJ, Azad RK, Raval A, Lawrence JG (2009) Detection of genomic islands via segmental genome heterogeneity. Nucleic Acids Res 37:5255–5266

Azad RK, Borodovsky M (2004) Effects of choice of DNA sequence model structure on gene identification accuracy. Bioinformatics 20:993–1005

Azad RK, Lawrence JG (2005) Use of artificial genomes in assessing methods for atypical gene detection. PLoS Comput Biol 1:e56

Azad RK, Lawrence JG (2007) Detecting laterally transferred genes: use of entropic clustering methods and genome position. Nucleic Acids Res 35:4629–4639

Azad RK, Lawrence JG (2011) Towards more robust methods of alien gene detection. Nucleic Acids Res 39:e56

Azad RK, Lawrence JG (2012) Detecting laterally transferred genes. Methods Mol Biol 855:281–308

Azad RK, Li J (2013) Interpreting genomic data via entropic dissection. Nucleic Acids Res 41:e23

Beiko RG, Charlebois RL (2007) A simulation test bed for hypotheses of genome evolution. Bioinformatics 23:825–831

Beiko RG, Hamilton N (2006) Phylogenetic identification of lateral genetic transfer events. BMC Evol Biol 6:15

Beiko RG, Ragan MA (2008) Detecting lateral genetic transfer : a phylogenetic approach. Methods Mol Biol 452:457–469

Beiko RG, Ragan MA (2009) Untangling hybrid phylogenetic signals: horizontal gene transfer and artifacts of phylogenetic reconstruction. Methods Mol Biol 532:241–256

Bejarano ER, Khashoggi A, Witty M, Lichtenstein C (1996) Integration of multiple repeats of geminiviral DNA into the nuclear genome of tobacco during evolution. Proc Natl Acad Sci U S A 93:759–764

Bergthorsson U, Adams KL, Thomason B, Palmer JD (2003) Widespread horizontal transfer of mitochondrial genes in flowering plants. Nature 424:197–201

Bininda-Emonds OR (2005) Supertree construction in the genomic age. Methods Enzymol 395:745–757

Bininda-Emonds OR, Sanderson MJ (2001) Assessment of the accuracy of matrix representation with parsimony analysis supertree construction. Syst Biol 50:565–579

Bock R (2010) The give-and-take of DNA: horizontal gene transfer in plants. Trends Plant Sci 15:11–22

Brown JR, Masuchi Y, Robb FT, Doolittle WF (1994) Evolutionary relationships of bacterial and archaeal glutamine synthetase genes. J Mol Evol 38:566–576

Chatterjee R, Chaudhuri K, Chaudhuri P (2008) On detection and assessment of statistical significance of Genomic Islands. BMC Genomics 9:150

Chen F, Mackey AJ, Stoeckert CJ Jr, Roos DS (2006) OrthoMCL-DB: querying a comprehensive multi-species collection of ortholog groups. Nucleic Acids Res 34:D363–D368

Christin PA, Edwards EJ, Besnard G, Boxall SF, Gregory R, Kellogg EA et al (2012) Adaptive evolution of C(4) photosynthesis through recurrent lateral gene transfer. Curr Biol 22:445–449

Day WH, McMorris FR (1992) Consensus sequences based on plurality rule. Bull Math Biol 54:1057–1068

Deschavanne PJ, Giron A, Vilain J, Fagot G, Fertil B (1999) Genomic signature: characterization and classification of species assessed by chaos game representation of sequences. Mol Biol Evol 16:1391–1399

Diao X, Freeling M, Lisch D (2006) Horizontal transfer of a plant transposon. PLoS Biol 4:e5

Do CB, Mahabhashyam MS, Brudno M, Batzoglou S (2005) ProbCons: Probabilistic consistency-based multiple sequence alignment. Genome Res 15:330–340

Dong J, Fernandez-Baca D, McMorris FR, Powers RC (2010) Majority-rule (+) consensus trees. Math Biosci 228:10–15

Dufraigne C, Fertil B, Lespinats S, Giron A, Deschavanne P (2005) Detection and characterization of horizontal transfers in prokaryotes using genomic signature. Nucleic Acids Res 33:e6

Durbin R, Eddy S, Krogh A, Mitchison G (1998) Biological Sequence Analysis: Probabilistic models of proteins and nucleic acids. Cambridge University Press, Cambridge, UK, p 350

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797

Efron B, Halloran E, Holmes S (1996) Bootstrap confidence levels for phylogenetic trees. Proc Natl Acad Sci U S A 93:13429–13434

Eulenstein O, Chen D, Burleigh JG, Fernandez-Baca D, Sanderson MJ (2004) Performance of flip supertree construction with a heuristic algorithm. Syst Biol 53:299–308

Felsenstein J (1989) PHYLIP: Phylogeny Inference Package (Version 3.2). Cladistics 5:164–166

Garcia-Vallve S, Romeu A, Palau J (2000) Horizontal gene transfer in bacterial and archaeal complete genomes. Genome Res 10:1719–1725

Gelman A, Rubin DB (1996) Markov chain Monte Carlo methods in biostatistics. Stat Methods Med Res 5:339–355

Gogarten JP (1995) The early evolution of cellular life. Trends Ecol Evol 10:147–151

Gogarten JP, Townsend JP (2005) Horizontal gene transfer, genome innovation and evolution. Nat Rev Microbiol 3:679–687

Gophna U, Charlebois RL, Doolittle WF (2006) Ancient lateral gene transfer in the evolution of Bdellovibrio bacteriovorus. Trends Microbiol 14:64–69

Goremykin VV, Salamini F, Velasco R, Viola R (2009) Mitochondrial DNA of *Vitis vinifera* and the issue of rampant horizontal gene transfer. Mol Biol Evol 26:99–110

Hayes WS, Borodovsky M (1998) How to interpret an anonymous bacterial genome: machine learning approach to gene identification. Genome Res 8:1154–1171

Hilario E, Gogarten JP (1993) Horizontal transfer of ATPase genes–the tree of life becomes a net of life. Biosystems 31:111–119

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755

Karlin S (1998) Global dinucleotide signatures and analysis of genomic heterogeneity. Curr Opin Microbiol 1:598–610

Katoh K, Asimenos G, Toh H (2009) Multiple alignment of DNA sequences with MAFFT. Methods Mol Biol 537:39–64

Kaundal R, Raghava GPS (2009) RSLpred: an integrative system for predicting subcellular localization of rice proteins combining compositional and evolutionary information. Proteomics 9:2324–2342

Kaundal R, Kapoor AS, Raghava GPS (2006) Machine learning techniques in disease forecasting: a case study on rice blast prediction. BMC Bioinforma 7:485

Kaundal R, Saini R, Zhao PX (2010) Combining machine learning and homology-based approaches to accurately predict subcellular localization in Arabidopsis. Plant Physiol 154:36–54

Keeling PJ, Palmer JD (2008) Horizontal gene transfer in eukaryotic evolution. Nat Rev Genet 9:605–618

Kishino H, Hasegawa M (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in hominoidea. J Mol Evol 29:170–179

Koonin EV, Makarova KS, Aravind L (2001) Horizontal gene transfer in prokaryotes: quantification and classification. Annu Rev Microbiol 55:709–742

Koulintchenko M, Konstantinov Y, Dietrich A (2003) Plant mitochondria actively import DNA via the permeability transition pore complex. EMBO J 22:1245–1254

Kurland CG (2005) What tangled web: barriers to rampant horizontal gene transfer. Bioessays 27:741–747

Kurland CG, Canback B, Berg OG (2003) Horizontal gene transfer: a critical view. Proc Natl Acad Sci U S A 100:9658–9662

Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J et al (2001) Initial sequencing and analysis of the human genome. Nature 409:860–921

Lapierre P, Lasek-Nesselquist E, Gogarten JP (2012) The impact of HGT on phylogenomic reconstruction methods. Brief Bioinform (in press)

Lawrence JG, Ochman H (1997) Amelioration of bacterial genomes: rates of change and exchange. J Mol Evol 44:383–397

Lawrence JG, Ochman H (1998) Molecular archaeology of the *Escherichia coli* genome. Proc Natl Acad Sci U S A 95:9413–9417

Lawrence JG, Ochman H (2002) Reconciling the many faces of gene transfer. Trends Microbiol 10:1–4

Logan DC (2006) Plant mitochondrial dynamics. Biochim Biophys Acta 1763:430–441
Médigue C, Rouxel T, Vigier P, Hénaut A, Danchin A (1991) Evidence of horizontal gene transfer in *Escherichia coli* speciation. J Mol Biol 222:851–856
Nakamura A, Schmitt M, Schmitt N, Simon HU (2005) Inner Product Spaces for Bayesian Networks. J Machine Learning Res 6:1383–1403
Nelson KE, Clayton RA, Gill SR, Gwinn ML, Dodson RJ, Haft DH et al (1999) Evidence for lateral gene transfer between Archaea and bacteria from genome sequence of Thermotoga maritima. Nature 399:323–329
Nesbo CL, Boucher Y, Doolittle WF (2001) Defining the core of nontransferable prokaryotic genes: the euryarchaeal core. J Mol Evol 53:340–350
Nguyen N, Mirarab S, Warnow T (2012) MRL and SuperFine+MRL: new supertree methods. Algorithms Mol Biol 7:3
Ochman H, Lawrence JG, Groisman E (2000) Lateral gene transfer and the nature of bacterial innovation. Nature 405:299–304
Olsen GJ, Woese CR (1993) Ribosomal RNA: a key to phylogeny. FASEB J 7:113–123
Pevsner J (2003) Bioinformatics and functional genomics. John Wiley & Sons, Hoboken, New Jerssey
Pible O, Imbert G, Pellequer JL (2005) INTERALIGN: interactive alignment editor for distantly related protein sequences. Bioinformatics 21:3166–3167
Poptsova M (2009) Testing phylogenetic methods to identify horizontal gene transfer. Methods Mol Biol 532:227–240
Ragan MA (2001) On surrogate methods for detecting lateral gene transfer. FEMS Microbiol Lett 201:187–191
Richards TA, Soanes DM, Foster PG, Leonard G, Thornton CR, Talbot NJ (2009) Phylogenomic analysis demonstrates a pattern of rare and ancient horizontal gene transfer between plants and fungi. Plant Cell 21:1897–1911
Richardson AO, Palmer JD (2007) Horizontal gene transfer in plants. J Exp Bot 58:1–9
Robinson DF, Foulds LR (1981) Comparison of phylogenetic trees. Math Biosci 53:131–147
Salzberg SL, Delcher AL, Kasif S, White O (1998) Microbial gene identification using interpolated Markov models. Nucleic Acids Res 26:544–548
Salzberg SL, White O, Peterson J, Eisen JA (2001) Microbial genes in the human genome: lateral transfer or gene loss? Science 292:1903–1906
Sanchez C (2011) Horizontal gene transfer: eukaryotes under a new light. Nat Rev Microbiol 9:228
Sandberg R, Winberg G, Branden CI, Kaske A, Ernberg I, Coster J (2001) Capturing whole-genome characteristics in short sequences using a naive Bayesian classifier. Genome Res 11:1404–1409
Schmidt HA, von Haeseler A (2007) Maximum-likelihood analysis using TREE-PUZZLE. Curr Protoc Bioinformatics. Chapter 6:Unit 6 6
Schmidt HA, Strimmer K, Vingron M, von Haeseler A (2002) TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics 18:502–504
Sheveleva EV, Hallick RB (2004) Recent horizontal intron transfer to a chloroplast genome. Nucleic Acids Res 32:803–810
Shimodaira H (2002) An approximately unbiased test of phylogenetic tree selection. Syst Biol 51:492–508
Shimodaira H, Hasegawa M (1999) Multiple Comparisons of Log-Likelihoods with Applications to Phylogenetic Inference. Mol Biol Evol 16:1114–1116
Simossis VA, Heringa J (2003) The PRALINE online server: optimising progressive multiple alignment on the web. Comput Biol Chem 27:511–519
Simossis VA, Heringa J (2005) PRALINE: a multiple sequence alignment toolbox that integrates homology-extended and secondary structure information. Nucleic Acids Res 33:W289–W294
Stanhope MJ, Lupas A, Italia MJ, Koretke KK, Volker C, Brown JR (2001) Phylogenetic analyses do not support horizontal gene transfers from bacteria to vertebrates. Nature 411:940–944

Stegemann S, Bock R (2009) Exchange of genetic material between cells in plant tissue grafts. Science 324:649–651

Stegemann S, Keuthe M, Greiner S, Bock R (2012) Horizontal transfer of chloroplast genomes between plant species. Proc Natl Acad Sci U S A 109:2434–2438

Suzuki K, Yamashita I, Tanaka N (2002) Tobacco plants were transformed by Agrobacterium rhizogenes infection during their evolution. Plant J 32:775–787

Swenson MS, Suri R, Linder CR, Warnow T (2012) SuperFine: fast and accurate supertree estimation. Syst Biol 61:214–227

Swofford D (1998) PAUP* 4.0 Beta version, phylogenetic analysis using parsimony (and other methods). Sinauer Associates, Inc., Sunderland, Massachusetts

Swofford D, Olsen GJO (1990) Phylogenetic reconstruction. In: Hillis DM, Moritz C (eds) Molecular systematics. Sinauer Associates, Inc., Sunderland, Massachusetts, pp 411–501

Syvanen M, Kado CI (1998) Horizontal Gene Transfer. Chapman & Hall, Lodon

Tatusov RL, Galperin MY, Natale DA, Koonin EV (2000) The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Res 28:33–36

Thomas CM, Nielsen KM (2005) Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat Rev Microbiol 3:711–721

Thompson JD, Gibson TJ, Higgins DG (2002) Multiple sequence alignment using ClustalW and ClustalX. Curr Protoc Bioinformatics. Chapter 2:Unit 2 3

Tsirigos A, Rigoutsos I (2005a) A new computational method for the detection of horizontal gene transfer events. Nucleic Acids Res 33:922–933

Tsirigos A, Rigoutsos I (2005b) A sensitive, support-vector-machine method for the detection of horizontal gene transfers in viral, archaeal and bacterial genomes. Nucleic Acids Res 33:3699–3707

Vaughn JC, Mason MT, Sper-Whitis GL, Kuhlman P, Palmer JD (1995) Fungal origin by horizontal transfer of a plant mitochondrial group I intron in the chimeric CoxI gene of Peperomia. J Mol Evol 41:563–572

Vernikos GS, Parkhill J (2006) Interpolated variable order motifs for identification of horizontally acquired DNA: revisiting the Salmonella pathogenicity islands. Bioinformatics 22:2196–2203

Vernikos GS, Parkhill J (2008) Resolving the structural features of genomic islands: a machine learning approach. Genome Res 18:331–342

Woese CR (1991) The use of ribosomal RNA in reconstructing evolutionary relationships among bacteria. In: Selander RK, Clark AG, Whittam TS (eds) Evolution at the Molecular Level. Sinauer Associates Inc., Sunderland, MA, pp 1–24

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains archaea, bacteria, and eucarya. Proc Natl Acad Sci U S A 87:4576–4579

Wolf YI, Rogozin IB, Grishin NV, Koonin EV (2002) Genome trees and the tree of life. Trends Genet 18:472–479

Woloszynska M, Bocer T, Mackiewicz P, Janska H (2004) A fragment of chloroplast DNA was transferred horizontally, probably from non-eudicots, to mitochondrial genome of Phaseolus. Plant Mol Biol 56:811–820

Won H, Renner SS (2003) Horizontal gene transfer from flowering plants to Gnetum. Proc Natl Acad Sci U S A 100:10824–10829

Chapter 12
Alien Gene Transfer: Challenges and Opportunities

Jitendra Kumar and Aditya Pratap

Abstract Alien genes have contributed several traits in the crop plants which are not available in the cultivated background. These have helped plant breeders in creating newer genetic diversity, thereby providing additional avenues of selection of better plant types. Vertical and horizontal transfer of alien genes has changed the fate of several crops by imparting resistance to diseases and insect–pests, tolerance to abiotic stresses such as salinity, water stress, and high temperature as well as improving quality. However, alien gene transfer is always not so easy and smooth as it appears from the successful examples of such transfers with massive effects in some crops, especially cereals. Several challenges such as pre- and post-fertilization barriers in distant crosses, problems in normal chromosome pairing, linkage drag, pleiotropic effects and role of recipient genome background on the expression of introgressed alien gene(s) in HGT and erratic regeneration protocols, difficulty in isolation of genes from wild species and their expression in recipient plants, and possibilities of gene flow from cultivated to wild types pose significant challenges to make alien gene transfer a routine process across all crop species. Nevertheless, refinements in various gene transfer technologies have led to generation of tremendous opportunities which provide newer means to obtain successful alien gene transfer in even those species which were earlier considered to be either recalcitrant to tissue culture or were considered as difficult. This chapter discusses in detail various challenges and opportunities associated with alien gene transfer in crop plants.

Keywords AB-QTL • Cisgenesis • Gene flow • Gene silencing • Intragenesis • Linkage drag • Pleiotropic effects • Reciprocal crossing

---

J. Kumar (✉) • A. Pratap, Ph.D.
Crop Improvement Division, Indian Institute of Pulses Research,
Kanpur, Uttar Pradesh 208 024, India
e-mail: jitendra73@gmail.com

A. Pratap and J. Kumar (eds.), *Alien Gene Transfer in Crop Plants, Volume 1: Innovations, Methods and Risk Assessment*, DOI 10.1007/978-1-4614-8585-8_12,
© Springer Science+Business Media New York 2014

12.1 Introduction

The influence of alien gene transfer on the way the fate of a crop can be changed was first demonstrated as early as in 1956 when a small segment from *Aegilops umbellulata* Zhuk. carrying a gene for resistance to leaf rust was translocated onto the wheat chromosome 6B (Sears 1956). This revolutionary work ushered an era of utilization of wild genetic resources for the improvement of crop plants. Consequently, interspecific and intergeneric hybridization have been widely adopted by plant breeders across different crops and used to develop improved cultivars with enhanced agronomic performance, resistance to biotic and abiotic stresses, and quality improvement. However, the success in utilizing wild genetic resources for transfer of desirable genes has not been uniform across all crop species since alien gene transfer largely depends upon availability of such resources, ease of hybridization and expression of the trait of interest in the progeny. Owing to these requirements, routine alien gene introgression from wild species is still a challenge before scientists for harnessing the desirable genes of wild species across all species. While evaluation of wild relatives and identification of genes conferring traits of interest themselves pose greatest challenges to breeders, effecting successful hybridization and obtaining a viable progeny with the gene(s) of interest transferred into them is further a difficult task. For involving wild species in hybridization, these need to be available in the vicinity of the recipient parent and their flowering must synchronize with that of the cultivated species. Raising wild species and exotic germplasm in field condition is often not easy and development of controlled conditions such as glass houses and plant growth chambers requires huge investments on time and money. Alien gene introgression is sometimes associated with several other difficulties such as linkage drag and pleiotropic effects; while in some instances this is associated with some unforeseen advantages also such as development of chromosome elimination technique for doubled haploidy breeding in wheat and barley. Advancements of in vitro techniques, in vivo and in vitro hormonal manipulations, techniques such as somatic hybridization and protoplast fusion and the most recent development of cisgenesis and intragenesis have offered commendable opportunities towards alien gene introgression. This chapter discusses the opportunities and challenges of alien gene transfer in crop plants.

12.2 Sources of Alien Genes and Their Characterization

The genes present in distant relatives (i.e., wild species) are usually known as alien genes. Therefore, these species are important source of such alien genes in crop plants. Among these species, phylogenetic relationships have been established on the basis of crossability of cultivated species with the wild species and other cytological and molecular analysis. This has led to characterization of wild species into

primary, secondary, and tertiary gene pools according to the gene pool concept of Harlan and De Wet (1971). This gene pool concept provides the knowledge regarding the possibilities of transferring alien genes controlling desirable traits from wild species either through conventional crossing or by using the advanced modern technologies. In general, species within the primary gene pool are easily crossable with each other and hence have been used easily for transfer of alien genes. Although wild species belonging to secondary gene pool may also cross readily with cultivated species, some post-zygotic barriers restrict their use in alien gene transfer. However as described earlier, recent advances in tissue culture techniques have made it feasible to use the species of this group. Wild species of tertiary gene pool are not found cross compatible with cultivated species. A large proportion of wild species belongs to this group and consequently is of no use for crop improvement through sexual manipulations (see Chap. 1 for details).

12.3 Challenges in Alien Gene Transfer

Alien gene introgression has opened new ways and opportunities in creating additional genetic variability and providing newer avenues of useful selection in crop plants besides helping in evolution of the crop species. While developments in hybridization strategies and advancement of in vitro techniques have made alien gene introgression in cultivated species easier, certain challenges are still there which make alien gene introgression a routine practice a bit difficult in plant breeding. Challenges and opportunities (discussed in next section) for exploitation of alien gene pools have been presented in Fig. 12.1.

12.3.1 Vertical Gene Transfer

12.3.1.1 Crossability Barriers

Wild species are an important reservoir of useful genes. However their use, especially of those species belonging to the tertiary gene pool, has been limited for transferring the useful genes due to crossability inhibition and limited recombination between chromosomes of wild and cultivated species (Brar and Khush 1986; Khush and Brar 1992; Sitch 1990). These crossability barriers developed during the process of speciation frustrate breeders' efforts in successful hybridization between species of different gene pools. The pre-fertilization cross incompatibility between parent species arises when pollen grains do not germinate, the pollen tube does not reach ovary, or the male gametes do not fuse with female gametes (Chowdhury and Chowdhury 1983; Shanmungam et al. 1983).

Fig. 12.1 Challenges and opportunities for exploitation of alien gene resources in crop improvement

12.3.1.2 Chromosome Pairing

Pairing of chromosomes of wild species with the cultivated species in their hybrids is the key to transfer of gene(s) across species. Genetic control of pairing of chromosomes derived from two different genomes has been identified in wheat, where this gene is known as *Ph1*. The suppressing of the *Ph1*-pairing regulation of polyploid wheats and oat has resulted in desired chromosome pairing and hence alien gene transfers into these crop species (Jauhar 2006). Such cytogenetic manipulations, including the suppression of the *Ph1* system, for recombining desirable alien chromatin into wheat were termed as chromosome engineering (Sears 1972). Essentially similar cytogenetic manipulations affecting gene transfer can also be done in hexaploid oats (Jellen and Leggett 2006). In rice, very limited chromosome pairing has been observed at metaphase I in F_1 hybrids of cultivated and wild species. Therefore, it has been difficult to transfer the alien genes from wild species to cultivated species (Brar and Khush 1997). Similarly, the high response of wheat to chromosome elimination technique for induction of haploids with respect to embryo formation has

been reported mainly due to genotypic nonspecificity, which is because of the insensitivity of the maize pollen to the action of *Kr1* and *Kr2* genes, which express in the style of many wheat varieties (Laurie and Bennett 1987a, b).

12.3.1.3 Linkage Drag

One of biggest challenges in using wild species for introgression of alien genes into cultivated background is the association of undesirable genes with the useful alien genes, known as linkage drag. Its effect is more severe in crops with diploid genetic systems because their genomes are more sensitive to genetic imbalance compared to relatively more buffered polyploid genomes. This has resulted in exploitation of only a few exotic genes in alien germplasm in agriculture (Friebe et al. 1996). In wheat, for example, genes other than the targeted gene (e.g., *Sr39* transferred from wild species '*Aegilops speltoides* Tauschii') was carried on the alien chromatin (Xu et al. 2008) during introgression, which had a deleterious effect on yield and quality (The et al. 1988; Lukaszewski 2000; Labuschagne et al. 2002). Therefore, it became important to eliminate the excess *Ae. speltoides* chromatin surrounding *Sr39* in order to make this gene useful for fighting against *Ug99* (Niu et al. 2011). The details about the impact of linkage drag have already been discussed in various chapters of this book.

12.3.1.4 Background Effects

It has been observed that there is a problem of variable expression of introgressed alien genes in cultivated backgrounds. In wheat, Chinese spring and *Leymus racemosus* translocated chromosomes carrying genes for resistance to *Fusarium* Head blight have been transferred to different common wheat backgrounds. However, expression of resistant gene was observed to be uniform among the resultant lines. These results demonstrated that effects of genetic backgrounds on the expression of alien resistance genes in wheat were due to epistatic interactions (Cai et al. 2005). Therefore, it is evident that efficient manipulation of alien chromatin and selection of proper recipient genotypes play a crucial role in the success of alien introgression for Fusarium head blight resistance (Stack et al. 2003; Garvin et al. 2003).

12.3.1.5 Pleiotropic Effects of Alien Genes

Sometimes introgressed alien genes affect more than one trait. If such effects are positively associated with desirable traits, introgression of alien genes with pleiotropic effects can be useful. However, if these are associated with undesirable traits, it becomes a challenge for breeders to use them in crop improvement. For example in wheat, introgression of leaf rust resistance gene *Lr47* (from *Triticum speltoides*) led to an overall reduction of 3.8 % in grain yield; nevertheless it varied significantly

across genotypes and environments. At the same time, lines with the alien *Lr47* segment showed consistent increase in grain and flour protein concentration, while there was significant decrease in flour yield and an increase in flour ash (Brevis et al. 2008). Similarly, the slow rusting genes in wheat often have pleiotropic effects on multiple rust diseases. Cloning of the well-characterized pleiotropic resistance gene *Lr34/Yr18/Sr57/Pm38/Sb1/Bdv1/Ltn1* showed that it belonged to the ABC-transporter group and was distinct from cloned race-specific resistance genes (Krattinger et al. 2009).

12.3.2 Horizontal Gene Transfer (HGT)

12.3.2.1 Regeneration Protocol

Horizontal transfer of alien genes across genera requires suitable regeneration system for the development of transgenic plants. This is one of the major challenges, especially in recalcitrant species such as food legumes that restrict transfer of alien genes through genetic transformation (i.e., transgenics). Tissue-culture techniques are part of a large group of strategies and range from molecular genetics to recombinant DNA studies, genome characterization, gene-transfer and in vitro regeneration of plants. All these tools require totipotent tissues that readily respond to tissue culture procedures. In most of the species, in vitro regeneration is highly genotype specific and cultivated varieties are rarely amenable to regeneration. Additionally, morphogenesis is generally very slow and very often there are problems like development of albinos and vitreous tissues, and no-response in dedifferentiated calli. Therefore, successful and reliable plant regeneration in many crop species still remains an aspiration that requires considerable refinement in technology and training of the human resources to develop the skills that are needed to generate green plants.

12.3.2.2 Isolation of Genes form Wild Species

Desirable genes present in the background of wild species belonging to primary gene pool have been exploited in development of improved varieties in several crops. These can be isolated through map-based cloning. However introgressions are accompanied by linkage drag where recombination is suppressed in the target gene region, and standard recombination-based approaches cannot be used in the molecular dissection of the target genes (Gill et al. 2011). In addition to this isolation of desirable genes from species belonging to secondary and tertiary gene pools is still difficult through map-based cloning. Although the wild species are good genetic resources for genes controlling resistance to biotic and abiotic stresses as well as quality traits, these could not be used extensively in breeding programs in either way.

12.3.2.3 Expression of Alien Genes

HGT trough genetic transformation is one of the most exciting approaches that opened practical opportunities for the improvement of crop plant without any limitation of genome boundaries. However, the unpredictable silencing or variable expression of transgenes is a ubiquitous phenomenon and it is an important challenge for genetic engineering of crops. This has been observed invariably in all plant species studied (Cerutti et al. 1997). There is not yet a reliable way to prevent silencing, although the converse affects—consistent gene silencing—has been reported (Angell and Baucombe 1997). Silencing resulting from interactions among multiple copies of transgenes and related endogenous genes involves homology-based mechanisms that act at either the transcriptional or post-transcriptional level (Meyer and Saedler 1996). It has been shown that high level expression of foreign proteins in plants often leads to gene silencing (Pickford and Cogoni 2013).

12.3.2.4 Gene Flow

Flow of alien transgenes from transgenic plants to their weedy and wild relatives through sexual reproduction and/or vegetative propagation is one of major concerns with potential ecological risks. Gene flow from the transgenic plants having resistance to diseases and insect-pests, drought and salt tolerance, and herbicide resistance can significantly enhance the ecological fitness of weedy and wild populations. As results, they can become aggressive weeds that can have unpredictable consequences to local ecosystems. This gene flow can also change the original wild populations and better ecological fitness could even lead to the extinction of endangered wild species populations locally (Kiang et al. 1979). Therefore, alien gene transfer through genetic transformation has a challenge of its negative impact of present ecological system.

12.4 Opportunities

During the past two decades, a lot of information relating to possible gene flow between cultivated crop species and their wild relatives, crossability barriers and methods to overcome them has been generated. These developments have provided tremendous opportunities to breeders to introgress alien genes into cultivated species. Simultaneously, the efforts in wide hybridization for transferring the alien gene into cultivated crops have also provided opportunities to develop new methods/techniques, which have made genetic enhancement of crop plants easier and faster. The following section discusses the new opportunities that have been generated while transferring alien genes from wild species.

12.4.1 Cross Compatibility

In has been reported that certain genes are responsible for pairing between the chromosomes leading to cross compatibility of wild species with the cultivated ones. In wheat, a gene *Ph1* has been identified on chromosome 6B, which suppresses the chromosome pairing. Suppression or removal of this chromosome pairing controlling gene has been used to transfer alien genes following wide hybridization coupled with the manipulation of chromosome pairing. This chromosome-mediated gene transfer has resulted in the development of several commercial cultivars with genes of alien origin. For example, a small segment from *Aegilops umbellulata* that carried a gene for resistance to leaf rust has been incorporated onto wheat chromosome 6B using cytogenetic techniques (Sears 1956). Subsequently, improved wheat cultivars with improved agronomic performance, pest tolerance, and high yields have been developed using inter-specific and inter-generic hybridization (Friebe et al. 1996; Jauhar and Chibbar 1999).

12.4.2 Advances in In Vitro Techniques

Concerted efforts has been made to develop the new technologies/ways in order to overcome the crossing barriers and avail the opportunity to transfer the alien gene(s) from cross-incompatible species/genera of crop plants. Major developments have been made in tissue culture techniques such as embryo rescue, ovule culture, and in vitro hormonal treatments, which have greatly increased the scope of distant hybridization in crop plants (Gupta and Sharma 2005; Clarke et al. 2006; Fratini and Ruiz 2006; Mallikarjuna et al. 2006). The embryo rescue technique has been successfully used for hybridization of cultivated lentil with *L. ervoides* and *L. nigricans* and a two-step in vitro method of embryo–ovule rescue has led to obtain successful distant hybrids (Cohen et al. 1984; Fiala 2006; Fratini and Ruiz 2006). Similarly in chickpea, embryo rescue technique has been successfully used to obtain viable embryos from *C. arietinum* × *C. bijugum* and *C. arietinum* × *C. pinnatifidum* crosses (Clarke et al. 2006). In rice, low crossability and abortion of hybrid embryos are the commonly occurring problems for crosses made between cultivated rice and distantly related wild species. However, use of embryo rescue produces F_1s and subsequent backcrossing of these F_1s resulted in fertile plants with normal diploid chromosome complement ($2n = 24$) or $2n = 25$ (monosomic alien addition lines). The fertile progenies were selfed to produce advanced introgression lines and evaluated for transfer of useful traits (Brar and Khush 1997).

Somatic hybridization using protoplast fusion has a great potential to overcome the barriers of gene transfer from wild species to cultivated species (Powers et al. 1976; Davey et al. 2005). Plants have been regenerated from protoplast cultures in *Pisum* (Ochatt et al. 2000), *Trifolium* (Gresshoff 1980), and *Melilotus* (Luo and Jia 1998). Asymmetric protoplast fusion has been used for *Medicago* improvement (Tian and Rose 1999). Initially, although, protoplast-derived tissues were obtained

in ricebean, no shoot regeneration could be obtained from these tissues. Later, shoot regeneration from protoplasts of *V. sublobata* was reported by Bhadra et al. (1994) and the maximum protoplast yield was obtained from 5-day-old seedlings. There are no reports of successful growth or regeneration of protoplasts of *Lens* species. Rozwadowski et al. (1990) cultured protoplasts from lentil epicotyl tissue and around 6 % of protoplasts developed into cell colonies.

12.4.3 Chromosome Doubling

Vertical transfer of alien genes through wide hybridization in some cases is restricted due to sterility of F_1 plants since this leads to problems in advancement of generation to F_2 for generating recombinants. This generally occurs in distant species where the parental species have different genomes. In such cases, no chromosome paring occurs in F_1 generation, which leads to sterility. However, studies have shown that chromosome doubling of either of the parental species before crossing or of the F_1 itself increases the chances of obtaining a viable hybrid. This has been used in several crop species including wheat, *Vigna*, cotton where allopolyploid species have been developed following chromosome doubling from most of the semi-fertile and completely seed sterile F_1 hybrids (Dana 1966; Pande et al. 1990). Thus the chromosome doubling technique has given additional opportunities to transfer alien genes using allopolyploids as a bridge species in wide crosses. In pigeonpea, chromosome doubling of diploid F_1 hybrids derived from cross between *Cajanus platycarpus* × *C. cajan* gave mature seeds in tertraploid F_1 hybrids and subsequent selfed generations. As the result, it was possible to introgress the resistance gene for *Phytophthora* blight disease from *C. platycarpus* (Mallikarjuna and Moss 1995). Irradiation techniques have also been successful in recovering fertile plants in F_1s and increased pod set in interspecific crosses between *V. radiata* × *V. umbellata* (Pandiyan et al. 2008).

12.4.4 Reciprocal Crossing

It has been observed that disharmony the between genome of one species and cytoplasm of the other causes fertilization barrier, restricting the transfer of alien genes from wild species. However, reciprocal crossing in such cases has given an opportunity of successful recovery of viable hybrids. This approach has been reported successful in crossing between *V. mungo* and *V. radiata*, where using the later species as female parent produced successful hybrids (Verma and Singh 1986; Ravi et al. 1987). Another species *V. riukinensis* if used as only a male parent with *V. angularis* and *V. umbellata* produced successful hybrids (Siriwardhne et al. 1991). As a general rule, use of species with higher chromosome number as the female parent is more successful compared to their reciprocals.

12.4.5 Use of Bridge Species

Wild species belonging to secondary and tertiary gene pools when directly involved in crossing with cultivated species do not result in fertile hybrids. However, use of another species which crosses easily with both, cultivated and wild species, can often be used as bridge species for transferring the alien genes. This approach has been successfully used in lentil where *L. ervoides* was used as a bridge species for transferring genes for resistance to ascochyta blight and anthracnose from *L. lamottei* and *L. nigricans* to *L. culinaris* following embryo rescue technique (Ye et al. 2002; Tullu et al. 2006). In mungbean, bruchid resistance has been transferred from *V. umbellata* to azuki bean by using the bridge species, *V. nakashimae* (Tomooka et al. 1992, 2000). Based on close relationship reported in perennial *Cicer anatolicum, C. reticulatum* and *C. echinospermum,* bridge cross approach deserves further attention. In buckwheat, *Fagopyrum homotropicum* (4×) has been used to improve the success of interspecific hybridization between the two cultivated buckwheat species, *Fagopyrum tataricum* and *F. esculentum* (Wang et al. 2002).

The successful crossing of wild species of barley, *Hordeum chilense* with diploid, tetraploid, and hexaploid wheats resulted in development of intergeneric amphiploids known as tritordeums. *H. chilense* and its amphiploids showed usefulness for several traits viz., resistance to rusts, powdery mildew, *Septoria tritici*, karnal bunt, smuts, aphids, nematodes, and certain degree of tolerance to drought and salt. Thus hexaploid tritordeum has been recommended for use as bridge species to introgress genetic material from wild barley into durum wheat (Ballesteros et al. 2000). Another genus, limegrass (*Leymus arenarius* L. Mollis), which can cross with cultivated common wheat (*Triticum aestivum*; *T. carthlicum*), has also been reported to be used as bridge species for transferring stress tolerance and disease resistance (Anamthawat-Jónsson 1995).

12.4.6 Use of Growth Hormones

Application of growth hormones such as gibberellic acid (GA), naphthalene acetic acid (NAA), kinetin, or 2, 4-D (dimethylamine), singly or mixed, has given an opportunity to make success in crossing of wild species where the hybrid embryos in distant crosses die due to their small size. Post pollination application of these growth regulators helps to maintain the developing seeds by facilitating division of hybrid zygote and endosperm. Use of growth regulators after pollination provided successful interspecific crosses in *Phaseolus* (Stalker 1980), *Cajanus* (Singh et al. 1993), and *Cicer* (Shiela et al. 1992). In chickpea, when growth regulators were applied to pollinated pistils, it prevented initial pod abscission and helped to save the aborting hybrid embryos by embryo rescue techniques (Mallikarjuna 1999).

In *Vigna* species *V. vexillata* is the closest to cowpea (*V. unguiculata*) and has high level of resistance to insect–pests. Various accessions of *V. vexillata* showed high levels of resistance to pod sucking bugs, flower thrips, *Maruca vitrata*, bruchid, and *Striga gesnerioides* (Fatokun et al. 1993). Richness of V. vexillata in these traits attracts legume breeders to make interspecific crosses between *V. vexillata* and cowpea. For this purpose, application of 2,4-D was observed most effective among the various hormones used. Sprays of this hormone at low concentrations (approximately 1.0 mg/l) before or after pollination promoted the retention of *V. vexillata* flowers pollinated with cowpea and subsequently the pods resulting from the cross-pollination (see Fatokun 2002)

12.4.7 Backcrossing

Utilization of alien genes is sometimes restricted due to the poor expression of desirable traits transferred from wild species, even though the wild species may have cross compatibility with the cultivated species. In such a case, backcrossing of F_1 hybrid with the cultivated species in early generation makes use of wild species possible. This approach has been used in pigeonpea where *Cajanus platycarpus* genome was introgressed into cultivated pigeonpea by backcrossing F_1 hybrids rescued through embryo culture followed by in vitro culture of aborting embryos of BC_1 progeny (Mallikarjuna et al. 2006). Similarly, one or more backcrosses with the recurrent parent are often required in common bean to restore the fertility of hybrids derived from crossing of cultivated species with *P. acutifolius* and *P. parvifolius*. Using *P. acutifolius* as female parent in initial F_1 cross, and/or first backcrossing of *P. vulgaris*×*P. acutifolius* hybrid with *P. acutifolius,* is often more difficult than using *P. vulgaris* as the female parent of the initial cross and backcrossing the interspecific hybrid with *P. vulgaris* (Mejia-Jimenez 1994). The choice of parents (Federici and Waines 1988; Mejia-Jimenez 1994) and use of the congruity backcross (i.e., backcrossing alternately to each species) over recurrent backcrossing (Haghighi and Ascher 1988; Mejia-Jimenez 1994) facilitate interspecific crosses of common and tepary beans, in addition to recovery of fertility and more hybrid progenies.

12.4.8 Use of Cytology and Advanced Genomics Tools in Isolation of Alien Genes

Alien gene introgressions are many times accompanied by linkage drag while recombination is suppressed in the target gene region and hence all genes on an arm are inherited as a single block. In wheat, gene *Pm21* found on chromosome arm of distant wheat relative *Dasypyrum villosum* (L.) Candargy (syn. *Haynaldia villosa*) showed resistant to all races of *Blumeria graminis* (DC.) Speer f. sp. *tritici* (Bgt).

However, isolation of this alien gene could not be possible due to inheritance of whole chromosome arm. Cao et al. (2011) used cytology and gene expression analysis strategy to isolate these genes. For this purpose, they first used microarray analysis to identify candidate genes induced on infection with Bgt. Radiation hybrid mapping was used to localize the *Pm21* gene on a segment using. Subsequently following molecular cytology techniques FISH used successfully to isolate this gene. Study showed that this gene encodes a serine threonine protein kinase which plays an important role in powdery mildew resistance. This was verified by transformation and virus-induced gene silencing (Gill et al. 2011).

12.4.9 Molecular Markers Aided Backcrossing (AB-QTL Strategy)

It has been shown that introgression of desirable genes from wild species carries the undesirable gene(s) also. However use of a novel breeding strategy known as AB-QTL (Advanced Backcross-Quantitative Trait Loci) helps minimize the negative effect of linkage drag associated with alien gene introgression (Tanksley and Nelson 1996). This technology has been used successfully in tomato, wheat, and rice. In this approach, molecular markers associated with improved background are used to genotype the advance backcross progenies (BC_2/BC_3). As a result, progenies having minimum linkage drag are selected on the basis of genome recovery of improved genotypes. For example, development of salt tolerant genotypes in rice by introgression of salt tolerance genes into high yielding varieties is often difficult through conventional breeding methods due to the unexpected linkage drag encountered in the progenies, which affects yield and grain quality characteristics of rice cultivars (Jeung et al. 2005; Yeo and Shon 2001). Recently three backcrosses have been used to transfer positive alleles of *Saltol* (salt tolerance) from genotype “FL478” in the background of improved cultivar “BT7”. The selected lines in BC_3F_1 with the *Saltol* alleles showed improved salt tolerance and agronomic performance similar to the original BT7 in the field. As the result, marker-assisted backcross breeding (i.e., AB-QTL) could help in removing the linkage drag (Linh et al. 2012).

12.4.10 Recombinant DNA Technology

This is a horizontal gene transfer approach which has been discussed in detail in Chap. 5. Traditional breeding for transferring a desired gene into a crop plant depends on the source of the gene and the evolutionary distance of that source with the recipient crop. If the source of a gene is available in less or distantly related wild species, belonging to the secondary or even tertiary gene pool, it may be difficult to transfer through traditional crossing and if at all possible, it may take 10–15 years

or even longer. In such case, Genetic engineering offers an excellent tool for asexually inserting well-characterized gene(s) of unrelated organisms into plant cells, which on regeneration produce sporophytes with the inserted gene(s) integrated into their genome. This process may take less than a year to about 18 months in some cases, thus accelerating the process of genetic improvement of crop plants. Moreover, this exciting technology allows access to unlimited gene pool without the constraint of sexual compatibility and genome boundary. Tremendous potential of genetic transformation through microprojectile bombardment has, for example, been demonstrated in wheat and other cereals (Jauhar and Chibbar 1999; Dahleen et al. 2001; Jauhar and Khush 2002; Altpeter et al. 2005). Many isolated plant genes are now being transferred between sexually incompatible plant species. In chickpea and pigeonpea, *Helicoverpa* pod borer is a major insect–pest for which no genetic solution has been reported till now. However, efforts are now underway towards development of *Helicoverpa* resistant transgenic lines in these two important grain legumes. The recent report of a *Bt* chickpea is an encouraging step toward improvement of food legumes for difficult traits such as pod borer resistance (Acharjee et al. 2010). Similar is the case with botrytis gray mold in chickpea and efforts are underway to construct a resistance locus against this disease. For gene introgression purposes, difficult species falling in tertiary and quaternary gene pools may turn out to be important sources of alien genes. For example, identification and cloning useful genes from *P. filiformis, P. angustissimus*, and *P. lunana* and successful regeneration and transformation of common bean may facilitate gene introgression in the future.

12.4.11 Cisgenesis/Intragenesis

Use of transgenic crop varieties, especially in food crops, is not accepted widely for commercial production due to introgression of genes from altogether unrelated species. One of the major concerns of the general public about transgenic crops relates to the mixing of genetic materials between species that cannot hybridize by natural means. To meet this concern, the two transformation concepts, cisgenesis and intragenesis, were developed as alternatives to transgenesis. Both concepts imply that plants must only be transformed with the genetic material derived from the species itself or from closely related species capable of sexual hybridization. Furthermore, foreign sequences such as selectable marker genes and vector-backbone sequences should be absent. Intragenesis differs from cisgenesis by allowing use of new gene combinations created by in vitro rearrangements of functional genetic elements. Several surveys show higher public acceptance of intragenic/cisgenic crops compared to transgenic crops. Thus, although the intragenesis and cisgenesis concepts were introduced internationally only 8–10 years back, several different traits in a variety of crops have already been modified using these methods. Such crops developed in apple, barley, and potato are now in field trials and two have pending applications for deregulation in USA and European countries. Currently, intragenic/cisgenic plants are regulated as transgenic plants worldwide.

However, as the gene pool exploited by intragenesis and cisgenesis are identical to the gene pool available for conventional breeding, less comprehensive regulatory measures are expected. The regulation of intragenic/cisgenic crops is presently under evaluation in the European Union and in the US regulators are considering if a subgroup of these crops should be exempted from regulation. Although, in Australia, a narrow group of cisgenic crops with genes introduced from the same species without T-DNA borders and other foreign DNA would not fall under Australian GM definition, no such crops have yet been dealt there (Lusser and Cerezo 2012). Nevertheless, it is possible that the intragenic/cisgenic route will be of major significance for future plant breeding (see Holme et al. 2013 for details).

12.4.12 Development of New Technologies

Distant hybridization between two different genera has also helped in generating new technologies also. For example, the production of wheat haploid plants through wide hybridization followed by chromosome elimination was first used successfully by crossing wheat with *Hordeum bulbosum*, commonly referred to as the *Bulbosum* technique (Barclay 1975). However, because of the present of the crossability inhibitor (*Kr*) genes that express in the style of many wheat genotypes and inhibit the growth of pollen tube of *H. bulbosum*, this technique had limited practical use. Later on wheat × maize hybridization was followed where no such limitation is encountered, since maize is insensitive to the action of dominant genes *Kr1* and *Kr2,* located on the long arm of the chromosomes 5B and 5A, respectively (Sitch et al. 1985; Laurie and Bennett 1987a, b). Subsequently, Laurie and Bennet (1988) developed first in vitro method to rescue the haploid embryo from wheat × maize crosses. Similarly another Gramineae genera, *Imperata cylindrica* was reported to be still more efficient for haploid induction through distant crosses in triticale (Pratap et al. 2005) and wheat (Choudhary et al. 2005).

12.5 Conclusions and Future Prospects

Hybridization followed by gene transfer between different crop species is not a new phenomenon and has been known to occur since thousands of years in nature. Species in nature have remained often incompletely isolated for millions of years after they had been formed. Therefore, while evolution of complete reproductive isolation occurring in them, there has been a limited quantity of gene flow between the evolving species. The consequences of such gene transfers have been multifarious including an increase in genetic variability, origin and transfer of adaptations, evolution of species and ecotypes, breakdown of isolating barriers, and promotion of colonization and dispersal (Abbott et al. 2003). Both vertical and horizontal gene transfers together have been vital to the overall evolution of crop plants. The vertical

gene transfers have been largely responsible for recombination of genes within a species. HGT has addressed the issues related to genome boundary limitations in alien gene transfers. Alien gene transfer by both methods has revolutionized the global agriculture and in cereals crops its use helped to develop the diverse genotypes against the major diseases. This could be possible due to the better understanding of underlying biological principles for successful alien gene transfer in crop plants. Tremendous developments in advanced technologies and basic knowledge of genetics and genomics have taken place towards addressing the challenges in accomplishing alien gene transfer. The most important opportunities, which helped to overcome the challenges of alien gene transfer, are development of in vitro techniques and efficient regeneration protocols, in vivo hormonal manipulations, stress pretreatments, and application of chemicals and reagents to accomplish hybridization. These opportunities resolved important challenges in order to make alien gene transfer successful in many agricultural crops, especially cereals. In spite of this, for use of alien gene transfer as a routine for improvement of crop plants, there are still a number of challenges ahead.

Use of horizontal transfer for alien genes poses important challenges including the production, management, and maintenance of GM crops, availability of genes from wild source, reliable regeneration protocol in recalcitrant crop species, and social acceptability of GM crops. Although flow of transgene from GM population to natural population can help in increasing the frequency of favorable alleles in the sink population, it may have harmful consequences in the absence of selection such as increased weediness and the increased likelihood of the extinction of wild relatives and exotic germplasm. Though conventionally transferred alien genes are more acceptable in cultivated background by the society than GM crops, use of alien genes from secondary and tertiary gene pool is still a challenge for plant breeders. However further advancement in genetics, genomics, and tissue culture technologies will certainly increase the possibility of introgression of favorable genes/QTL for yield and other desirable traits from wild species and will help to widen the genetic base. As the result, we can achieve a sustainable growth in production and productivity of crop plants in changing environmental conditions.

References

Abbott RJ, James JK, Milne RI, Gillies ACM (2003) Plant introductions, hybridization and gene flow. Philos Trans R Soc Lond B Biol Sci 358:1123–1132

Acharjee S, Sarmah BK, Kumar PA, Olsenc K, Mahon R, Moar MJ, Moor A, Higgins TJV (2010) Transgenic chickpeas (*Cicer arietinum* L.) expressing a sequence-modified *cry2 A* gene. Plant Sci 178:333–339

Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, Daniell H, Datta K, Datta S, Dix PJ, Fauquet C, Huang N, Kohli A, Mooibroek H, Nicholson L, Nguyen TT, Nugent G, Raemakers K, Romano A, Somers DA, Stoger E, Taylor N, Visser R (2005) Particle bombardment and the genetic enhancement of crops: myths and realities. Mol Breed 15:305–327

Anamthawat-Jónsson K (1995) Wide-hybrids between wheat and lymegrass: breeding and agricultural potential. ICEL Agr Sci 9:101–113

Angell S, Baulcombe D (1997) Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. EMBO J 16:3675–3684

Ballesteros J, Cabrera A, Hernández P, Martín A, Ramírez MC, Rubiales D (2000) Prospect for the use of *Hordeum chilense* in durum wheat breeding. In: Royo C, Nachit M, Di Fonzo N, Araus JL (eds) Durum wheat improvement in the Mediterranean region: new challenges. CIHEAM, Zaragoza, pp 111–115

Barclay IR (1975) High frequencies of haploid production in wheat (*Triticum aestivum*) by chromosome elimination. Nature 256:410–411

Bhadra SK, Hammatt N, Paner JB, Davey MR (1994) A reproductive response for plant regeneration from seedling hypocotyl protoplasts of *Vigna sublobata* L. Plant Cell Rep 14:175–179

Brar DS, Khush GS (1986) Wide hybridization and chromosome manipulation in cereals. In: Evans DH, Sharp WR, Ammirato PV (eds) Handbook of plant cell culture, vol 4, Techniques and applications. MacMillan Publish Co, New York, pp 221–263

Brar DS, Khush GS (1997) Alien introgression in rice. Plant Mol Biol 35:35–47

Brevis JC, Chicaiza O, Khan IA, Jackson L, Morris CF, Dubcovsky J (2008) Agronomic and quality evaluation of common wheat near-isogenic lines carrying the leaf rust resistance gene *Lr47*. Crop Sci 48:1441–1451

Cai X, Chen PD, Xu SS, Oliver RE, Chen X (2005) Utilization of alien genes to enhance Fusarium head blight resistance in wheat – A review. Euphytica 142:309–318

Cao A, Xing L, Wang X, Yang X, Wang W, Sun Y, Qian C, Ni J, Chen Y, Liu D, Wang X, Chen P (2011) Serine/threonine kinase gene *Stpk- V*, a key member of powdery mildew resistance gene *Pm21*, confers powdery mildew resistance in wheat. Proc Natl Acad Sci USA 108:7727–7732

Cerutti H, Johnson A, Gillham N, Boynton J (1997) Epigenetic silencing of a foreign gene in nuclear transformants of *Chlamydomonas*. Plant Cell 9:925–945

Chaudhary HK, Sethi GS, Singh S, Pratap A, Sharma S (2005) Efficient haploid induction in wheat by using pollen of *Imperata cylindrica*. Plant Breed 124:93–95

Chowdhury RK, Chowdhury JB (1983) Compatability between *Vigna radiate* (L.) Wilczek and *Vigna umbellata* (Thumb) Ohwi and Ohashi. Genetica Agraria 37:257–266

Clarke HJ, Wilson JG, Kuo I, Lülsdorf MM, Mallikarjuna N, Kuo J, Siddique KHM (2006) Embryo rescue and plant regeneration in vitro of selfed chickpea (*Cicer arietinum* L.) and its wild annual relatives. Plant Cell Tissue Organ Cult 85:197–204

Cohen D, Ladizinsky G, Ziv M, Muehlbauer FJ (1984) Rescue of interspecific Lens hybrids by means of embryo culture. Plant Cell Tiss Org Cult 3:343–347

Dahleen LS, Okubara PA, Blechl AE (2001) Transgenic approaches to combat Fusarium head blight in wheat and barley. Crop Sci 42:628–637

Dana S (1966) Cross between *Phaseolus aureus* and *P. mungo*. Genetica 37:259–274

Davey MR, Anthony P, Power JB, Lowe KC (2005) Plant protoplasts: status and biotechnological perspectives. Biotechnol Adv 23:131–171

Fatokun CA (2002) Breeding cowpea for resistance to insect pests: attempted crosses between cowpea and Vigna vexillata. In: Fatokun CA, Tarawali SA, Singh BB, Kormawa PM, Tamò M (eds) Challenges and opportunities for enhancing sustainable cowpea production. Proceedings of the world cowpea conference III, international institute of tropical agriculture (IITA), Ibadan, Nigeria, pp. 52–61

Fatokun CA, Danesh D, Young ND, Stewart EL (1993) Molecular taxonomic relationships in the genus *Vigna* based on RFLP analysis. Theor Appl Genet 86:97–104

Federici CT, Waines JG (1988) Interspecific hybrid compatibility of selected *Phaseolus vulgaris* L. lines with *P. acutifolius* A. Gray, P. *lunatus* L., and P. *filiformis* Benthum. Annual Reporter Bean Improvement Cooperation 31:201–202

Fiala JV (2006) Transferring resistance to *Colletotrichum truncatum* from wild lentil species to cultivated lentil species (*Lens culinaris* subsp *culinaris*). MSc thesis, University of Saskatchewan, Saskatoon, Canada, pp.131

Fratini R, Ruiz ML (2006) Interspecific hybridization in the genus *Lens* applying *in vitro* embryo rescue. Euphytica 150:271–280

Friebe B, Jiang J, Raupp WJ, McIntosh RA, Gill BS (1996) Characterization of wheat alien translocations conferring resistance to diseases and pests: current status. Euphytica 91:59–87

Garvin DF, Stack RW, Hansen JM (2003) Genetic analysis of extreme Fusarium head blight susceptibility conferred by a wild emmer chromosome. In: Proc 10th international wheat genet symposium, Paestum, Italy, pp. 1139–1141

Gill BS, Friebe BR, White FF (2011) Alien introgressions represent a rich source of genes for crop improvement. Proc Natl Acad Sci U S A 108:7657–7658

Gresshoff PM (1980) *In vitro* culture of white clover, callus, suspension, protoplast culture and plant regeneration. Bot Gaz 141:157–164

Gupta D, Sharma SK (2005) Embryo-ovule rescue technique for overcoming post-fertilization barriers in interspecific crosses of *Lens*. J Lentil Res 2:27–30

Haghighi KR, Aschar PD (1988) Fertile intermediate hybrid between *Phaseolus vulgaris* L. and *P. acutifolius* from congruity backcrossing. Sex Plant Reprod 1:51–58

Harlan JR, De Wet MJ (1971) Towards a rational classification of crop plants. Taxonomy 20:509–517

Holme IB, Wendt T, Holm PB (2013) Intragenesis and cisgenesis as alternatives to transgenic crop development. Plant Biotechnol J 11:395–407

Jauhar PP (2006) Cytogenetic architecture of cereal crops and their manipulation to fit human needs: opportunities and challenges. In: Singh RJ, Jauhar PP (eds) Genetic resources, chromosome engineering, and crop improvement, vol 2, Cereals. CRC Taylor & Francis Press, Boca Raton, FL, pp 1–25

Jauhar PP, Chibbar RN (1999) Chromosome-mediated and direct gene transfers in wheat. Genome 42:570–583

Jauhar PP, Khush GS (2002) Importance of biotechnology in global food security. In: Lal R et al (eds) Food security and environmental quality in the developing world. CRC Press, Boca Raton, FL, pp 107–128

Jellen EN, Leggett JM (2006) Cytogenetic manipulation in oat improvement. In: Singh RJ, Jauhar PP (eds) Genetic resources, chromosome engineering, and crop improvement, vol 2, Cereals. CRC Taylor & Francis Press, Boca Raton, FL, pp 199–231

Jeung JU, Hwang HG, Moon HP, Jena KK (2005) Fingerprinting temperate japonica and tropical indica rice genotypes by comparative analysis of DNA markers. Euphytica 146:239–251

Khush GS, Brar DS (1992) Overcoming the barriers in hybridization. Theor Appl Genet (Monograph No. 16):47–61

Kiang YT, Antonvics J, Wu L (1979) The extinction of wild rice (*Oryza perennis formosa*) in Taiwan. J Asian Ecol 1:1–9

Krattinger SG, Lagudah ES, Spielmeyer W, Singh RP, Huerta-Espino J, McFadden H, Bossolini E, Selter LL, Keller B (2009) A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. Science 323:1360–1363

Labuschagne MT, Pretorius ZA, Grobbelaar B (2002) The influence of leaf rust resistance genes *Lr29, Lr34, Lr35* and *Lr37* on breadmaking quality in wheat. Euphytica 124:65–70

Laurie DA, Bennett MD (1987a) The effect of crossability loci *Kr1* and *Kr2* on fertilization frequency in haploid wheat × maize crosses. Theor Appl Genet 73:403–409

Laurie DA, Bennett MD (1987b) Wide crosses involving maize (*Zea mays*). Annual report of the plant breeding institute, pp. 66–68

Laurie DA, Bennett MD (1988) The production of wheat haploid plant from wheat × maize crosses. Theor Appl Genet 76:393–397

Linh LH, Linh TH, Xuan TD, Ham LH, Ismail AM, Khanh TD (2012) Molecular breeding to improve salt tolerance of rice (*Oryza sativa* L.) in the red river delta of Vietnam. Int J Plant Genomics 2012:9. doi:10.1155/2012/949038

Lukaszewski AJ (2000) Manipulation of the 1RS.1BL translocation in wheat by induced homoeologous recombination. Crop Sci 40:216–225

Luo JP, Jia JF (1998) Plant regeneration from callus protoplasts of the forage legume *Astragalus adsurgens* Pall. Plant Cell Rep 17:313–317

Lusser M, Cerezo ER (2012) Comparative regulatory approaches for new plant breeding techniques: workshop proceedings. JRC Technical Report EUR 25237 EN. European Commission. Joint Research Centre (2012). Luxembourg: Publications Office of the European Union. http://ftp.jrc.es/EURdoc/JRC68986.pdf, pp. 1–35

Mallikarjuna N (1999) Ovule and embryo culture to obtain hybrids from interspecific incompatible pollinations in chickpea. Euphytica 110:1–6

Mallikarjuna N, Moss JP (1995) Production of hybrids between *Cajanus platycarpus* and *C. Cajan*. Euphytica 83:43–46

Mallikarjuna N, Jadhav D, Reddy P (2006) Introgression of *Cajanus platycarpus* genome into cultivated pigeonpea, *C. Cajan*. Euphytica 149:161–167

Mejía-Jiménez A (1994) Interspecific hybridization between common and tepary beans: increased hybrid embryo growth, fertility, and efficiency of hybridization through recurrent and congruity backcrossing. Theor Appl Genet 88:324–331

Meyer P, Saedler H (1996) Homology-dependent gene silencing in plants. Annu Rev Plant Physiol Plant Mol Biol 47:23–48

Niu Z, Klindworth DL, Friesen TL, Chao S, Jin Y, Cai X, Xu SS (2011) Targeted introgression of a wheat stem rust resistance gene by DNA marker-assisted chromosome engineering. Genetics 187:1011–1021

Ochatt SJ, Mousset-Declas C, Rancillac M (2000) Fertile pea plants regenerate from protoplast when calluses have not undergone endoreduplication. Plant Sci 156:177–183

Pande K, Raghuvanshi SS, Prakash D (1990) Induced high yielding amphiploid of *Vigna radiata* × *V. mungo*. Cytologia 55:249–253

Pandiyan M, Ramamoorthi N, Ganesh SK, Jebaraj S, Pagarajan P, Balasubramanian P (2008) Broadening the genetic base and introgression of MYMV resistance and yield improvement through unexplored genes from wild relatives in mungbean. Plant Mutation Reports 2:33–38

Pickford AS, Cogoni C (2013) RNA-mediated gene silencing. Cell Mol Life Sci 60:871–882

Powers JB, Frearson EM, Hayward C, George D, Evans PK, Berry SF, Cocking EC (1976) Somatic hybridization of *Petunia hybrid* × *P. parodii*. Nature 263:500–502

Pratap A, Sethi GS, Chaudhary HK (2005) Relative efficiency of different Gramineae genera for haploid induction in triticale and triticale × wheat hybrids through the chromosome elimination technique. Plant Breed 124:147–153

Ravi J, Singh JP, Minocha, JL (1987) Meiotic behaviour of interspecific hybrids of *Vigna radiata* × *V. mungo*. In: Proceedings of the first symposium on crop improvement, Tamil Nadu Agricultural University, Coimbatore, India, pp. 58–59

Rozwadowski KL, Saxena PK, King J (1990) Isolation and culture of *Lens culinaris* Medik. Plant Cell Tissue Org Cult 15:175–182

Sears ER (1956) The transfer of leaf-rust resistance from *Aegilops umbellulata* to wheat. Brookhaven Symp Biol 9:1–22

Sears ER (1972) Chromosome engineering in wheat. In: Kimber G, Redei GR (eds) Stadler Symp., vol 4. University of Missouri, Columbia, pp 23–38

Shanmungam AS, Rathnaswamy R, Rangasamy SR (1983) Crossability studies between green gram and black gram. Curr Sci 52:1018–1020

Shiela VK, Moss JP, Gowda CLL, Rheenen HA (1992) Interspecific hybridization between *Cicer arietinum* and wild *Cicer* species. Int Chickpea Newslett 27:11–13

Singh J, Sidhu PS, Verma MM, Gosal SS, Singh J (1993) Wide cross hybridization in *Cajanus*. Crop Improv 20:27–30

Siriwardhane D, Egawa Y, Tomooka N (1991) Cross-compatibility of cultivated adzuki bean (*Vigna angularis*) and rice bean (*V. umbellata*) with their wild relatives. Plant Breed 107:320–325

Sitch LA (1990) Incompatibility barriers operating in crosses of *Oryza sativa* with related species and genera. In: Gustafson JP (ed) Genetic manipulation in plant improvement II. Plenum Press, New York, pp 77–94

Sitch LA, Snape JW, Firman SJ (1985) Intrachromosomal mapping of crossability gene sin wheat (*Triticum aestivum* L.). Theor Appl Genet 70:309–314

Stack RW, Frohberg RC, Hansen JM, Mergoum M (2003) Transfer and expression of resistance to Fusarium head blight from wild emmer chromosome 3A to bread wheat. In: Canty SC, Lewis J, Ward RW (eds) National Fusarium head blight forum proc, U.S. wheat and barley scab initiative, pp. 232 (abstract)

Stalker HT (1980) Utilization of wild species for crop improvement. Adv Agron 33:111–147

Tanksley SD, Nelson JC (1996) Advanced back cross QTL analysis, a method for the simultaneous discovery and transfer of valuable QTLs from unadapted germplasm into elite breeding lines. Theor Appl Genet 92:191–203

The TT, Latter BDH, McIntosh RA, Ellison FW, Brennan PS et al (1988) Grain yields of near-isogenic lines with added genes for stem rust resistance. In: Miller TE, Koebner RMD (eds) Proceedings of the 7th international wheat genetics symposium, vol 2, Cambridge, UK, pp. 901–906

Tian D, Rose RJ (1999) Asymmetric somatic hybridization between the annual legumes *Medicago truncatula* and *Medicago scutellata*. Plant Cell Rep 18:989–996

Tomooka N, Lairungruang C, Nakeeraks P, Egawa Y, Thavarasook C (1992) Development of bruchid-resistant mungbean using wild mungbean germplasm in Thailand. Plant Breed 109:60–66

Tomooka N, Kashiwaba K, Vaughan D, Ishimoto M, Egawa Y (2000) The effectiveness of evaluating wild species, searching for sources of resistance to bruchid beetle in the genus *Vigna* subspecies *Caratotropis*. Euphytica 115:27–41

Tullu A, Buchwaldt L, Lulsdorf M, Banniza S, Barlow B, Slinkard AE, Sarker A, Tar'an TD, Warkentin TD, Vandenberg A (2006) Sources of resistance to anthracnose (*Colletotrichum truncatum*) in wild *Lens* species. Genet Resour Crop Evol 53:111–119

Verma RPS, Singh DP (1986) Problems and prospects of interspecific hybridization involving green gram and black gram. Indian J Agr Sci 56:535–537

Wang Y, Scarthi R, Campbell C (2002) Interspecific hybridization between *Fagopyrum tataricum* (L.) Gaertn. and *F. esculentum* Moench. Fagopyrum 19:31–35

Xu SS, Dundas IS, Pumphrey MO, Jin Y, Faris JD et al (2008) Chromosome engineering to enhance utility of alien derived stem rust resistance. In: Appels R, Eastwood R, Lagudah E, Langridge P, Mackay M, McIntyre, Sharp (eds) Proceedings of the 11th international wheat genetics symposium, vol 1, Sydney University Press, Sydney, pp. 12–14

Ye G, McNiel DL, Hill GD (2002) Breeding for resistance to lentil ascochyta blight. Plant Breed 121:185–191

Yeo US, Shon JK (2001) Linkage analysis between some agronomic traits and resistance gene to brown planthopper in rice. Korean J Plant Breed 33:287–293

About the Editors

Dr. Aditya Pratap, born on October 18, 1976, is currently working as a Senior Scientist (Plant Breeding) in the Crop Improvement Division, Indian Institute of Pulses Research, Kanpur. He obtained his master's and Ph.D. degrees in Plant Breeding and Genetics from CSK Himachal Pradesh Agricultural University, Palampur, India in 1999 and 2003. Holding a brilliant academic and service record, he has been associated with crop research since last 10 years and has worked on genetic improvement of crop plants including wheat, triticale, rapeseed-mustard, chickpea and *Vigna* species and has been instrumental in the development of haploidy breeding protocol in cereals through chromosome elimination technique. He has been associated with the development and release of five crop varieties including two in rapeseed-mustard (RSPT2 and RSPR03), two in green gram (IPM 02-14 and IPM 02-3) and one in facultative winter wheat (Him Pratham) and registered two extra early maturing green gram genotypes (IPM 205-7 and IPM 409-4) while a few other varieties are in pipeline. His research interests include distant hybridization, doubled haploidy breeding, plant tissue culture, and molecular breeding. To his credit, he has about 100 publications which include research papers published in high-impact journals, technical bulletins, as well as reviews/chapters for best international publishers including Springer, Academic Press, CABI and CRC. He has published two books entitled, "Haploidy breeding in Triticale and triticale × wheat hybrids: Comparison of Anther Culture and Chromosome Elimination Techniques" by Lambert Academic Publishing, Germany and another on "Biology and Breeding of Food Legumes" published by CAB International, Oxfordshire, UK. He is also a recipient of the prestigious Norman E. Borlaug International Agricultural Science and Technology Fellowship. He is an acknowledged speaker and has several awards to his credit.

Dr. Jitendra Kumar, born in 1973, is presently working as Senior Scientist in the Division of Crop Improvement at Indian Institute of Pulses research, Kanpur. He has an excellent research career throughout. He secured Gold Medal during master's programme and pursued his Ph.D. in Genetics & Plant Breeding from G. B. Pant University

A. Pratap and J. Kumar (eds.), *Alien Gene Transfer in Crop Plants, Volume 1: Innovations, Methods and Risk Assessment*, DOI 10.1007/978-1-4614-8585-8,
© Springer Science+Business Media New York 2014

of Agriculture & Technology, Pantnagar, India. He was awarded CSIR-Research Associateship during 2003–2005 for postdoctoral studies at the Institute of Integrative Medicine, Jammu (India). He has more than 12 years research experience in genetic improvement using both conventional and molecular marker-assisted breeding approaches on various crops including medicinal and aromatic, cereal and pulse crops. During this period, he undertook study tours in Austria, Syria and Bangladesh. His research interests include conventional and molecular breeding, QTL analysis and marker-assisted selection for crop improvement. He has about 80 publications including research articles in reputed national and international journals, reviews, book chapters, popular articles, meeting reports, bulletins, *etc*. He has also co-edited the book "Biology and Breeding of Food Legumes" published by CAB International, Oxfordshire, UK. He has been associated with development of a high-yielding variety (IPL 316) of lentil and several others are in pipeline. His current priorities include involvement of molecular marker technology in conventional lentil breeding programme for making genetic improvement towards the biotic and abiotic stresses.

Index

A
Abiotic stresses
  drought, 122
  osmolytes, 122
  receptor molecules, 123
  transcription factors, 122, 123
  transgenic groundnut plants, 122
AB-QTL. *See* Advanced Backcross-Quantitative Trait Loci (AB-QTL)
AB-QTL analysis
  alleles and donor genes, 178
  application, 220
  crop plants, 179, 219
  maize, 221
  mapping study, 219–220
  molecular breeding approach, 178
  rice, 220
Advanced backcross (AB) population
  barley, 221
  cotton, 221–222
  crop plants, 217, 218–219
  epistasis interaction, 219
  genes/QTL, 217
  IL and CSSL, 221–222
  maize, 221
  NAM population, 226
  naturally introgressed lines, 225–226
  rice, 220
  RICLs, RICSLs and RCSLs, 223–224
  tomato, 219–220
  wheat, 221
Advanced Backcross-Quantitative Trait Loci (AB-QTL), 300
Agrobacterium-mediated transformation
  adventitious shoot production, 108
  agrolistics, 114
  antibiotics, 112
  anti-necrotic treatments, 110–111
  A. tumefaciens, 108
  desiccation, explants, 110
  floral-dip method, 114
  inoculation and co-culture medium, 111–112
  osmotic treatment, 109–110
  pre-conditioning and co-cultivation time, 110
  production, compounds, 108
  SAAT, 113
  selectable marker, 112–113
  surfactants, 111
  temperature, 111
  tissue culture technology, 108
  transfer, T-DNA, 109
  vacuum infiration method, 114
Alien gene
  introgression
    crop plants genetic variation, 166
    GSV, 167–168
    late blight, potato, 168
  MABC, 173–176
  mapping and transfer, AB-QTL analysis, 178–179
  mapping/tagging, 170
  molecular markers, 173
  wild species and linkage drag
    chickpea, 178
    compensating transfer, 176
    rice, 177–178
    wheat, 177
Alien introgression
  AB-population (*see* Advanced backcross (AB) population)

A. Pratap and J. Kumar (eds.), *Alien Gene Transfer in Crop Plants, Volume 1: Innovations, Methods and Risk Assessment*, DOI 10.1007/978-1-4614-8585-8,
© Springer Science+Business Media New York 2014

Alien introgression (*cont.*)
  "allele replacement", 234
  biotic and abiotic stresses, 212
  "breeding by design", 234
  "cryptic introgressions", 212
  "cytogenetics era", 212
  diversity sources
    "gene pool", 212, 214
    GP-1, 214–215
    GP-2, 215
    GP-3, 215–216
    GP-4, 216
  exotic germplasm (*see* Exotic germplasm, breeding)
  gene pools, exotic, 212, 213
  genotyping platforms, 233
  monocultures, 212
  morphological traits, 211
  Neolithic era, 211
  rice germplasm accessions, 233
  tools and techniques, 216–217
  Ug99, 212
Amphidiploid
  anti-microtubule drug, 30
  colchicine, 30
  cytological processes, 31
  FDR, 31
  F_1 hybrids, 30
  genetic studies, 33
  SDR, 32
  spontaneous chromosome doubling, 30
  synthesizing DHs (SynDH), 31
  UMCD, 31, 32
  unreduced gametes, 31, 32

B

Backcross breeding
  abiotic stress resistance, 226–227
  biotic stress resistance, 228, 229–230
  mineral nutrients, 232–233
  nutritional quality, 231–232
  "reverse breeding", 222
  "smart breeding", 222
  yield and yield contributing traits, 228, 230–231
Bayesian method, 274, 281
Bioinformatics approaches
  bacterial and plant genomes, 277
  canonical organismal phylogeny, 271
  caveats, 277
  chloroplast genome, 269
  and EEEP method, 278
  and HGT (*see* Horizontal gene transfer (HGT))
  human genome project, 277
  and IGT, 268
  mitochondria, 269
  nuclear genes, 269
  organismal tree, 278
  orthologous genes, 271
  pairwise sequence, 277
  parametric methods (*see* Parametric methods)
  phylogenetic tree (*see* Phylogenetic tree)
  plant-fungi HGT, 278
  prioritizing genes, 278
  psbA gene, 269
  rps11 gene, 269
  self-splicing intron, 269
  unusual phyletic pattern, 276–277
  viral DNAs and RNA, 268
Biolisitcs
  agrolistics approach, 114
  applicability and efficiency, 115
  co-transformation system, 116
  GUS expression, 115
  particle bombardment technique, 114
Biotic stresses
  fungus and bacteria resistance, 125–126
  insect resistance, 124
  traits, 123–124
  virus resistance, 124–125
Bootstrapping methods
  ancestral genome, 275
  bifurcating and multi-furcating branches, 274
  gene transfer, 275
  incongruent gene phylogenies, 275, 276
  non-trivial issue, 275
  "null" datasets, 275
  organismal tree, 274–275
  "orphan" genes, 276
  phylogenetic methods, 275
  Robinson-Foulds (R-F) distance, 275
Bottom up parametric methods
  description, 279
  gene clustering methods, 279–280
  non-clustering methods, 280–281
Bulbosum method, 148–149

C

Callus cultures
  Arabidopsis, 49
  DNA vector application method, 52–53
  donor plant effect, 49
  genotype *vs.* transformation efficiency, 51–52
  indirect organogenesis, 50

lipoic acid, 50
meristemoid, 48
micropropagation, 48
organ primordia, 48
PGRs, 49
picloram, 50
somatic embryogenesis, 50
TDZ, 50
transformation protocols, 51
zygotic embryo, 51
Cereals
Avena sativa L. (oat), 84
embryo rescue, 85–86
Hordeum vulgare L. (barley), 84
Oryza sp. (rice), 87
sorghum L. and *Zea mays* L. (corn), 87
Triticosecale (triticale), 87
Challenges and opportunities
AB-QTL strategy, 300
backcrossing, 299
bridge species, 298
chromosome pairing, 292–293
cisgenesis/intragenesis, 301–302
crop improvement, 291, 292
cross compatibility, 296
cultivated species, 295
cytology and genomics tools, 299–300
distant hybridization, 302
doubling, chromosome, 297
gene pools, 290–291
GM crops, 303
growth hormones, 298–299
HGT, 294–295
hybridization, 290
in vitro techniques, 296–297
introgression, 291
progeny, 290
reciprocal crossing, 297
recombinant DNA technology, 300–301
vertical gene transfer, 291–294
wheat chromosome 6B, 290
wild species, 290, 291
Chromosome elimination
DH, 147
hybrid embryo, 148
maize, 150
potato haploidy breeding, 158
wide crossing, 148
Chromosome manipulation
alien species, 27
classification, 26
elimination and haploid crop (*see* Haploid crop)
F_1 hybrid, 27–28
homoeologous pairing and translocation lines, 33–35
types, 27
unreduced gametes and amphidiploid (*see* Amphidiploid)
wide/distant hybridization, 26
Chromosome pairing, 292–293
Chromosome segment substitution line (CSSL), 221–222
Cisgenesis
gene introgression, 290
gene pools, 301–302
intragenesis, 301
transgenesis, 301
Clusters of Orthologous Groups (COGs), 273
Co-transformation, 116
Crop evolution, alien gene transfer
breeders and geneticists, 2
breeding, 17
detection, 13–15
distant hybridization, 15
gene flow, 15
genetic diversity, 16
genetic variation, 3
global crop production, 2
HGT (*see* Horizontal gene transfer (HGT))
hybridization (*see* Hybridization)
intragenesis and cisgenesis, 12–13
introgression, 16
methods, 4
natural introgressions, 4
reproductive barriers, 7–8
somatic hybridization, 10–12
"super plant types", 17
transgenics, 2–3
undesirable effects, 3
VGT, 5–6
CSSL. *See* Chromosome segment substitution line (CSSL)
Cytogenetics. *See* Molecular cytogenetics

D

DH. *See* Doubled haploidy (DH)
Direct organogenesis
genome stability and transformation, 47–78
induction medium, 47
PGRs, 46, 47
protocols, 47
SEs, 46
tissue culture protocols, 46
WPM, 47

Distant hybridization. *See also* Chromosome manipulation
  domesticated crop plants, 15
  and doubled haploidy breeding
    breeding programmes, 144
    cellular mechanism, 148
    conventional and DH breeding approaches, 146–147
    crop improvement, 143
    cytogenetic manipulations, 145
    DH (*see* Doubled haploidy (DH))
    interspecific hybridization, 144–145
    reproductive barriers, 146
    wide crosses, 144
    wild relatives, 145
  pre-and post-fertilization barriers, 7
Domestication
  genetic bottlenecks, 220
  non-domestication species, 233
  polyploidy, 212
  wild plants, 211–212
Doubled haploidy (DH)
  Hordeum vulgare × *H. bulbosum*, 148–149
  Oat × maize, 57–158
  Solanum tuberosum × *S. phureja*, 158
  Triticale × Imperata cylindrica, 156–157
  Triticum aestivum × *H. bulbosum*, 149–150
  Wheat × *Imperata cylindrica*, 155–156
  Wheat × Job's Tears, 154
  Wheat × Maize, 150–153
  Wheat × Pearl Millet, 153–154
  Wheat × *Tripsacum dactyloides*, 153

E
EDF. *See* Extended DNA fiber (EDF)
Efficient evaluation of edit paths (EEEP) method, 278
Embryogenesis
  Agrobacterium, 47
  callus, 46
  cryopreservation, 55
  lipoic acid, 46
  small cell volumes, 49
  somatic (*see* Direct organogenesis)
  suspension cultures, 54–56
  zygotic, 45
Embryo rescue
  breeding program, 99
  cereals, 84–87
  horticultural species, 94–989
  legumes, 87–94
  medium components, 98
  oil crops (*see* Oil crops)
  post-fertilization barriers, 78
Exotic germplasm, breeding
  abiotic stress resistance, 226–227
  bio-fortification, 232–233
  biotic stress resistance, 228, 229–230
  nutritional quality, 231–232
  yield and yield contributing traits, 228, 230–231
Extended DNA fiber (EDF), 198–200

F
FHB. *See Fusarium* head blight (FHB)
First division restitution (FDR), 31
FISH. *See* Fluorescent in situ hybridization (FISH)
Flippase/flippase recombination target (FLP/Frt), 118
Fluorescent in situ hybridization (FISH)
  advantages, 191
  DNA mapping technique, 191
  and GISH, 193
  transgene behaviour, 192
Fusarium head blight (FHB), 228

G
Gene dispersal, 248, 250
Gene flow, 13, 15, 295, 302
Gene flow impact, GM crops
  commercial transgenic crops, 255
  experimental studies, 255–256
  herbicide and insect resistances, 255
  mechanisms
    baseline information, 248–250
    environmental and ecological factors, 250–251
    human-aided dispersal mechanisms, 248
    transgenic, 247
  non-GMvarieties, 248, 251
  phenotypes, 254
  probability
    experimental data, 251
    field studies, 251–253
    "gene deletor" technology, 253
    physical barriers, 253
    quantification, 254
    transgene mitigation, 254
  risk assessment
    conventional varieties, 256
    generate information, 256
    post release monitoring, 257
    quantification, 256–257

Gene introgression, 31
Gene mapping, 175
Gene pool
  crossability inhibitor *(Kr)* genes, 217
  cytogenetic procedures, 216
  genetic diversity, 212, 213
  GP-1, 214–215
  GP-2, 215
  GP-3, 215–216
  GP-4, 216
  traits, 221
Gene silencing, 295, 300
Genetically modified (GM) crops
  agricultural plants, transgene, 248
  case-specific factors, 248
  decision making process, 258
  description, 247
  environmental damage, 258
  gene flow (*see* Gene flow)
  inevitability, 258
  transgenic plants, 258
Genetic manipulations (GM), 126, 303
Genetic transformation
  biosafety issue, 126
  development, transgenic plants, 106
  GM crops, 126
  HGT, 127
  marker free plants (*see* Marker free transgenics)
  plant gene transfer methods (*see* Plant gene transfer methods)
  plant regeneration, tissue cultures, 106–107
  stress tolerance, crop species (*see* Stress tolerance, crop species)
  transformation vectors, 107
  transgenic technologies, 106
Genomic in situ hybridization (GISH)
  chromosome, 193–194
  description, 193
  and FISH, 195
  hybrid nature confirmation, 194
  metaphase spreads, 193, 194
  molecular cytogenetics, 196
  polyploid species, 193
GISH. *See* Genomic in situ hybridization (GISH)
Glucuronidase (GUS), 115
Glyphosate oxidoreductase (GOX), 113
GM. *See* Genetic manipulations (GM)
GOX. *See* Glyphosate oxidoreductase (GOX)
GP-1. *See* Primary gene pool (GP-1)
GP-2. *See* Secondary gene pool (GP-2)
GP-3. *See* Tertiary gene pool (GP-3)
GP-4. *See* Quaternary gene pool (GP-4)
Grassy stunt virus (GSV), 167–168
GSV. *See* Grassy stunt virus (GSV)
GUS. *See* Glucuronidase (GUS)

H

Haploid. *See* Doubled haploidy (DH)
Haploid crop
  Arabidopsis thaliana plants, 29–30
  centromere-specific histone CENH3, 29, 30
  gene introgression, 28
  interspecific hybridization, 28
  uniparental/paternal chromosomes, 29
Herbicide resistance
  gene pool, 257
  GM and non-GM varieties, 254–255, 257
  in North America, 255
HGT. *See* Horizontal gene transfer (HGT)
High resolution mapping, 201
Hordeum vulgare × *H. bulbosum*, 148–149
Horizontal gene transfer (HGT)
  Agrobacterium, 8
  conjugation, 270
  de novo chloroplast, 269
  expression, alien genes, 295
  gene flow, 295
  genetic transformation (*see* Genetic transformation)
  LGT, 8
  man-made, 268–269
  mitochondria, 9
  mitochondrion-mitochondrion, 269
  Mu-like element (MULE), 9
  plants, 268, 282
  prokaryotic and eukaryotic genome evolution, 268, 283
  quantification, 271
  regeneration protocol, 294
  transduction, 270
  transformation, 270
  transgene introgression, 9–10
  wild species, 294
Horticultural species
  Allium (onion), 94
  Capsicum baccatum (chilli pepper), 94
  Citrus species, 94, 97
  Cucumis sativus L. (cucumber), 97
  embryo rescue, 95–96
  Lycopersicon esculentum Mill. (tomato), 97
  Prunus species, 97
  Solanum tuberosum subsp. *tuberosum* (potato), 98
  Vitis vinifera L. (grape), 98

Hybridization
chromosome pairing, 6
gene transfer, 7
sexually compatible species, 6
somatic, 10–12

I
IGT. *See* Intracellular gene transfer (IGT)
IL. *See* Introgression line (IL)
In situ hybridization (ISH) techniques
plant chromosomes, 191
radioactive, 197
Intracellular gene transfer (IGT), 268
Intragenesis
cisgenesis, 301
gene introgression, 290
gene pools, 301–302
transgenesis, 301
Introgression
hybrid generations, 16
introgression lines (ILs), 14
transgene, 9–10
Introgression line (IL), 221–222
In-vitro plant regeneration
callus culture (*see* Callus cultures)
cell suspension cultures (*see* Suspension cultures)
direct organogenesis, 46–48
protoplasts, 59–61
tissue target, 44–45
transformation and transgenic, 45
ISH. *See* In situ hybridization (ISH) techniques

K
Karyotype
chromosomes and development, 189
diagnostic change, 200
wheat telosomes, 200

L
Lateral gene transfer (LGT), 8
Legumes
Arachis hypogaea L. (peanut), 87–88
Cajanus cajan L. (pigeonpea), 88
Cicer arietinum L. (chickpea), 88
embryo rescue of legume species, 89–91
Glycine max L. Merr. (soybean), 88, 92
Lens culinaris Medik. (lentil), 92
Lupinus sp. (lupin), 92
Medicago sativa L. (alfalfa), 92–93
Phaseolus vulgaris L. (dry bean), 93
Trifolium sp. (clover), 93
Vigna sp., 93–94
LGT. *See* Lateral gene transfer (LGT)
Linkage drag
gene introgression, 290, 299, 300
progenies, 300
wild species, 293

M
MALD. *See* Mapping by admixture linkage disequilibrium (MALD)
Mapping by admixture linkage disequilibrium (MALD), 225
Marker-assisted backcrossing (MABC)
cryptic alien introgression, 175
Lr58 gene, 175–176
molecular markers, 174
rice, 174
wheat, 174–175
Marker free transgenics
autoexcision strategy, 121
chemical-inducible system, 119–120
co-transformation, 116
Cre/loxP system, 117–118
FLP/Frt recombination system, 118
heat-inducible system, 120
MAT vector system, 116–117
negative selection system, 121
positive selection system, 120–121
R/RS recombination system, 118–119
site-specific recombination, 117
SMG, 115
transformation events, 115–116
transposon-based marker methods, 119
Markov Chain Monte Carlo (MCMC), 274
MAT. *See* Multi-autotransformation (MAT)
MFISH. *See* Multicolour FISH (MFISH)
Microspore suspension cultures
auxin analogues, 58
DH, 58
gametic embryos, 57–58
genetic transformation, 59
haploid cells, 57, 58
in vitro androgenesis, 59
regeneration conditions, 58
starvation stress, 58
transgenic, 59
Molecular cytogenetics
biotic and abiotic stresses, 187–188
conventional, 188–189
EDF, 198–200
FISH, 191–193

flow cytometry, 200–201
GISH, 193–196
ISH techniques, 189–190
MFISH, 197
nuclei FISH, 197–198
plant breeding tools, 187
Molecular markers
alien gene (*see* Alien gene)
alleles/QTL, 170, 173
classification, 168–169
crop domestication, 166
description, 166
high-throughput array, 169
MABC, 173–176
mapping/tagging, alien genes, 170–172
NGS methods, 170
screening techniques, 169
Multi-autotransformation (MAT), 116–117
Multicolour FISH (MFISH), 197

N
Nested association mapping (NAM) population, 226
Next-generation sequencing (NGS), 170
NGS. *See* Next-generation sequencing (NGS)
Non-GM varieties
gene flow, 254
hybridisation, 248, 255
transgenic line, 254

O
Oat × maize, 157–158
Oil crops
Brassicaeae
B. napus / B. juncea, 78
embryo culture, 79
embryo rescue, 80–82
ovary/ovule culture, 79
pistil/ovary-ovule method, 83
placenta/ovule-embryo culture, 83
siliques, 83
Helianthus annuus L., 80–82, 84

P
Parametric methods
application, 278
bottom up (*see* Bottom up parametric methods)
inferences, 282
recipient genome, 278
top down, 281–282
PGRs. *See* Plant growth regulators (PGRs)
Phylogenetic tree
bootstrapping methods (*see* Bootstrapping methods)
construction methods, 273–274
DNA and RNA, 272
gene flow, 272
HGT, 272
identification, 272–273
multiple sequence alignment, 273
nucleotide/amino acid substitution, 273
orthologous gene trees, 272
vertical inheritance, 272
Plant gene transfer methods
Agrobacterium-mediated gene transfer (*see Agrobacterium*-mediated transformation)
biolistics, 114–115
DNA delivery protocols, 115
Plant growth regulators (PGRs), 49
Plant regeneration
genetic transformation process, 44
in-vitro (*see In-vitro* plant regeneration)
protocols, 44
tissue cultures
totipotent cells, 107
transformation, plants, 106
Pleiotropic effects, 293–294
Primary gene pool (GP-1), 214–215
Protocols. *See* Embryo rescue
Protoplasts
cell wall, 60
electrofusion and electroporation, 60
enzymatic digestion, 60
fusion, protoplast, 60
microinjection, 61
plasma membrane, 59
suspension cultures, 59
totipotency and durability, 59

Q
Quaternary gene pool (GP-4), 216

R
Reciprocal crossing, 297
Recombinant chromosome substitution lines (RCSLs), 223–224
Recombinant inbred chromosomal lines (RICLs), 223–224
Recombinant inbred chromosome substitution lines (RICSLs), 223–224

Recombinase/recognition sites (R/RS), 118–119
Reproductive barriers, 7–8

S
Secondary gene pool (GP-2), 215
Second division restitution (SDR), 32
Selectable marker
  GOX gene, 113
  phosphomannose isomerase, 113
  plant transformation vectors, 107
  prokaryotic, 107
  transgenic plants, 119
Selectable marker gene (SMG), 115
SEs. *See* Somatic embryos (SEs)
SMG. *See* Selectable marker gene (SMG)
Solanum tuberosum × *S. phureja*, 158
Somaticembryos(SEs), 46
Somatic hybridization, 10–12
Sonication-assisted *Agrobacterium*-mediated transformation (SAAT), 113
Stress tolerance, crop species
  abiotic stresses, 122–123
  biotic stresses, 123–126
Suspension cultures
  A. tumefaciens, 56
  biolistic transformation, 57
  composition, liquid medium, 54
  cryopreservation, embryogenic, 55
  definition, 53
  dicotyledonous and monocotyledonous plants, 56
  embryogenic cell suspensions, 56
  genetic transformation, 53
  in vitro cultured cells, 53
  microprojectile bombardment, 57
  microspore, 57–59
  morphogenic potential, 54
  organogenesis, 55
  plant tissues, 53
  silicon carbide (SiC) whiskers, 57
  transformation target, 55–56

T
TALE. *See* Transcription activator like effector (TALE)
TDZ. *See* Thidiazuron (TDZ)
Tertiary gene pool (GP-3), 215–216
Thidiazuron (TDZ), 50
Top down parametric methods, 281–282
Transcription activator like effector (TALE), 234
Transformation
  Agrobacterium, 52, 56
  biolistic protocol, 53
  cell suspension, 55–56
  efficiency, callus, 51–52
  micropropagation procedures, 48
  morphogenetic, 50
  plant genetic, 44
  protocols, 46, 51
  SEs, 48
  suspension cultures, 56–57
Transformation vectors, 107
Transgene
  agricultural plants, 248
  escape, 126, 127
  introgression, 9–10
  mitigation, 254
  organicand conventional fields, 258
Translocation lines, 33–35
Triticale × *Imperata cylindrica*, 156–157
Triticum aestivum × *H. bulbosum*, 149–150

U
UMCD. *See* Unreductional meiotic cell division (UMCD)
Unreductional meiotic cell division (UMCD), 31, 32
UPGMA methods, 273

V
Vertical gene transfer
  crossability barriers, 291–292
  effects, genetic backgrounds, 293
  linkage drag, 293
  pleiotropic effects, 293–294
Vertical gene transfer (VGT), 5–6
VGT. *See* Vertical gene transfer (VGT)

W
Wheat × *Imperata cylindrica*, 155–156
Wheat × Job's Tears, 154
Wheat × Maize
  chromosome elimination system, 150
  embryogenesis, 151
  hexaploid triticale, 152
  hybrid embryos and endosperms, 151
  hybrid zygotes, 150
  pollen source, haploid induction, 153
Wheat × Pearl Millet, 153–154
Wheat × *Tripsacum dactyloides*, 153
Wide hybridization, 148, 156, 157

Wild relatives
Ae. geniculata, 227
exotic germplasm, 212
fertility barriers, crop plants, 212
genes/QTL introgression, 217
genetic variation, 217
maize, 232
Woody plant medium (WPM), 47
WPM. *See* Woody plant medium (WPM)

Z

Zink-finger (ZF), 234