Jameel M. Al-Khayri · Shri Mohan Jain
Dennis V. Johnson *Editors*

Advances in Plant Breeding Strategies: Agronomic, Abiotic and Biotic Stress Traits

Volume 2

Springer

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Preface

Thus far conventional plant breeding methods have been successfully used for sustainable food production worldwide. Human population is increasing at an alarming rate in developing countries, and food availability to feed the additional mouths could gradually become a serious problem. Moreover, agriculture production is being adversely affected as a result of environmental pollution, rapid industrialization, water scarcity, erosion of fertile topsoil, limited possibility of expanding arable land, lack of improvement of local plant types, erosion of genetic diversity, and dependence on a relatively few crop species for the world's food supply. According to FAO, 70 % more food must be produced over the next four decades to feed the projected 9 billion people by the year 2050. Only 30 plant species are used to meet 95 % of the world's food requirements, which are considered as the *major crops*. The breeding programs of these crops have been very much dependent on the ready availability of genetic variation, either spontaneous or induced. Plant breeders and geneticists are under constant pressure to sustain and expand food production by using innovative breeding strategies and introducing minor crops which are well adapted to marginal lands and provide a source of nutrition as well as crops having abiotic and biotic stress tolerances. In traditional breeding, introgression of one or a few genes in a cultivar is carried out via backcrossing for several generations. Now, new innovative additional plant breeding tools, including molecular breeding and plant biotechnology, are available to plant breeders, which have a great potential to be used along with the conventional breeding methods for sustainable agriculture. With the development of new molecular tools such as genomics, molecular marker-assisted backcrossing has made possible rapid introgression of transgenes, reduction of linkage drag, and manipulation of genetic variation for the development of improved cultivars. For example, molecular breeding has great potential to become a routine standard practice in the improvement of several crops. However, a multidisciplinary approach of traditional plant breeding, plant biotechnology, and molecular biology would be strategically ideal for developing new improved crop varieties worldwide to feed the world. This book highlights the recent progress in the development of plant biotechnology, molecular tools, and their usage in plant breeding.

The basic concept of this book is to examine the use of innovative methods augmenting traditional plant breeding toward the development of new crop varieties, grown under different environmental conditions, to achieve sustainable food production.

This book consists of two volumes: Volume 1 subtitled *Breeding, Biotechnology and Molecular Tools* and Volume 2 subtitled *Agronomic, Abiotic and Biotic Stress Traits*. This volume contains 18 chapters highlighting breeding strategies for specific plant traits including improved nutritional and pharmaceutical properties as well as enhanced tolerance to insects, diseases, drought, salinity, and temperature extremes expected under global climate change. Chapters addressing these topics are grouped into four parts: Part I Sustainability, Nutrition and Pharmaceuticals; Part II Forage and Tree Traits; Part III Abiotic Stress Tolerance; and Part IV Biotic Stress Resistance.

Each chapter begins with an introduction covering related backgrounds and provides in-depth discussion of the subject supported with high-quality color photos, illustrations, and relevant data. The chapter concludes with prospects for future research directions and a comprehensive list of pertinent references to facilitate further reading.

The book is an excellent reference source for plant breeders and geneticists engaged in breeding programs involving biotechnology and molecular tools together with traditional breeding. It is suitable for both undergraduate and postgraduate students specializing in agriculture, biotechnology, and molecular breeding, as well as for agricultural companies.

Chapters were written by internationally reputable scientists and subjected to a review process to assure quality presentation and scientific accuracy. We greatly appreciate all chapter authors for their participation toward the success and quality of this book. We are proud of this diverse collaborative undertaking, especially since the two volumes represent the efforts of 105 scientists from 29 countries. We are also grateful to Springer for giving us an opportunity to compile this book.

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Part I
Sustainability, Nutrition and Pharmaceuticals

Chapter 1
Sustainable Agriculture and Plant Breeding

Dinesh Narayan Bharadwaj

Abstract World population is expected to increase from the current 6.7 billion to more than 10 billion people by the year 2050. This 45 % increase in the current world population will create demand for increased food and other raw materials. At present the supply of fossil fuel, fertilizers, water and chemicals such as insecticides, pesticides and fungicides are at their peak; but this situation will not remain linear in the future. Modern agriculture is essentially based on varieties bred for high performance under high-input systems which generally do not perform well under low-input conditions. Excessive uses of these inputs are posing serious threats to ecology, environment, soil health and ground water. Furthermore, the amount of arable land for crop cultivation is limited and decreasing due to urbanization, salinization, desertification and environmental degradation. With respect to global warming, yields of important food, feed and fiber crops will decline. In addition to these environmental factors, abiotic and biotic stresses also cause losses to crop production. Thus, the challenge before agriculture scientists is to improve the genetic architecture of agricultural crops to perform well against threats and stresses; this will require diverse approaches to enhance the sustainability of agriculture farms. This proposed shift in plant breeding goals, from high energy input and high performance of agriculture, entails an improved rationalization between yield and energy coupled with high quality food as global resources. Sustainable crop production is a way of growing food in an ecologically and ethically responsible manner that does not harm the environment and sustains communities.

Keywords Biodiversity • Ecology • Environment • Global resources • Plant genetic resources • Scarcity • Sustainable agriculture • Renewal energy

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1.1 Introduction

Since the advance of human civilization, agriculture has continued to be the backbone of the economy all over the globe, as most people depend upon the agriculture sector for food, feed, shelter and clothes. The current agriculture sector has sufficient production of food grains, oilseeds, pulses, fiber and other commodities to feed the global population and provide raw materials for industrial use.

After World War II, the development of improved technology, new high-yielding varieties, mechanization, enhanced use of chemical and fertilizers led to maximizing yields (Rosset et al. 2000), but this development adversely affected agroecology and labor demands in agriculture. Present agricultural industrialization to produce the food, fiber and other commodities has significantly reduced the risk of cost-input in both sectors. Owing to advanced technology and the Green Revolution during the past 50 years, agricultural production has nearly tripled. Modern agriculture is essentially based on plant varieties for high performance under a high-input system of fertilizer, water, pesticides and fossil fuels. Currently the use of these chemical inputs is at their peak because these varieties do not perform well under low input conditions. However, on one hand, the availability of these inputs will not continue forever and on the other hand, the situation is alarming because of the degradation of natural resources and ecosystems. The Green Revolution came at a high cost to the environment, public health and social welfare. High agricultural production also maximized the risk of depletion of topsoil, contamination of ground water, decrease in the number of family farms and ecological imbalances. The current farming system is dependent on fossil fuels and toxic inputs; ignorance of their hazards has proved to be a dead-end road. The increase in the population growth coupled with urbanization and industrialization has led to the decrease in per capita availability of land for cultivation, availability that continues to diminish day by day, as a result the global resources are in decline and human population is increasing.

As projected to 2050, world population will reach around >10 billion people (Dasgupta 1998), this increased population will create a huge demand for food and raw materials to provide basic human needs (Table 1.1). The most debatable topic of today is land degradation, environmental losses due to maximum use of harmful

Table 1.1 Trends in increase of population, food production, resource consumption and future demands

	Years		
Resources	1960	2000	2050
Population (billions)	3	6	9–10
Food production (mt)	1.8 × 109	3.5 × 109	6.5 × 109
Agricultural water (km^3)	1,500	7,130	12–13.500
N fertilizer use (Tg)	12	88	120
P fertilizer use (Tg)	11	40	55–60
Pesticide use (Tg, active ingredient)	1.0	3.7	10.1

agriculture inputs, which are steadily degrading soil fertility, contaminating ground water, exacerbating ecological imbalances, intensifying emission of harmful greenhouse gases and promoting a rise of atmospheric temperature. These changes began a key shift in the natural climate cycle and result in excessive rain, snowfall, drought, wind storms and other calamities affecting human society. Overall crop productivity is also declining due to soil degradation in the form of low nutrition, reduced water-holding capacity, toxicity, infestation and persistence of weeds. To overcome these socioeconomic problems, several countries have started a new movement known as *sustainable agriculture* which is now receiving increasing support and acceptance of intellectuals from various sectors of agriculture and industry. Sustainable agriculture has been gearing up with many environmental and social concerns and offers innovative and economically-viable opportunities for farmers, laborers, consumers, policymakers and industrialist in the entire societal food chain.

1.2 Conventional Plant Breeding

Conventional agricultural methods are heavily dependent on non-renewable energy (fossil fuel) resources. Plant breeding adapts the principles of genetics to improve the quality, diversity and high yield performance of crops with the objective of better-suited plants to human needs. Plant breeding is simply the process of crossing the best parent species, identifying and recovering the best performing progeny over the parents. The process of plant breeding involves three steps: germplasm collections, identification of superior phenotypes and their hybridization to develop improved cultivars (Fehr 1987; Stoskopf et al. 1993). Furthermore, the best plants are selected from the mixed population; they are then stabilized for several years. These newly-developed improved varieties with superior traits are then tested in different climates for their suitability, multiplied and distributed among farmers, but the process may take 12–15 years. In this regard the Veery variety of wheat is a good example of plant breeding for it was developed through 3,170 crosses involving 51 crop parents collected from 21 countries (Rajaram et al. 1996).

1.3 Sustainable Agriculture: Concepts and Principles

The manifold progress in the agricultural sector during the past half-century in crop and livestock productivity has been strongly driven by over exploitation of resources such as fertilizers, irrigation, agricultural machinery, pesticides and land use efficiency. It would be overly-optimistic to assume that these resources will also remain linear in future (Tilman 1999). The concerns of sustainability in agricultural system center on the need to develop technologies and practices that do not have adverse or harmful effects on the environment, food quality, productivity and services to farmers and their society. New approaches are required to integrate biological and

ecological concerns into food production, minimize the use of non-renewable inputs that causes harm to the environment or to the human health (farmers and consumers), which can make productive use of the knowledge and skill of farmers to solve common agricultural and natural resource problems. Improving natural capital is a central theme of sustainable agriculture and can make the best use of available resources, genotypes of crops, animals and the balanced ecological conditions under which they are sustained (Cassman et al. 2002). Agricultural sustainability suggests a focus on genotype improvement through complete exploitation of modern biological approaches, understanding the benefits of ecological and agronomic management, manipulation and redesign. The ecological management of agro-ecosystems that addresses energy flows, nutrient cycling, population-regulating mechanisms and system resilience can lead to the redesign agriculture at a site specific magnitude (Pretty 2008). Sustainable agriculture outcomes can be positive for food quality, productivity, reduced chemicals use and carbon balances (Roberts and Lighthall 1993). Significant challenges, however, remain to develop national and international policies to support the wider emergence of more sustainable forms of agricultural production across both developed and developing countries. The sustainable agriculture development program recognizes use of limited natural resources, economic growth and encourages saving them for future use. It gives due consideration to long-term interests in preserving topsoil, biodiversity and rural communities, rather than short-term profit interests. It is a site specific and dynamic process with a system-wide holistic approach in solving farm management problems (Fig. 1.1). Sustainable agriculture has three main goals: environmental health, economic profitability and socioeconomic equity.

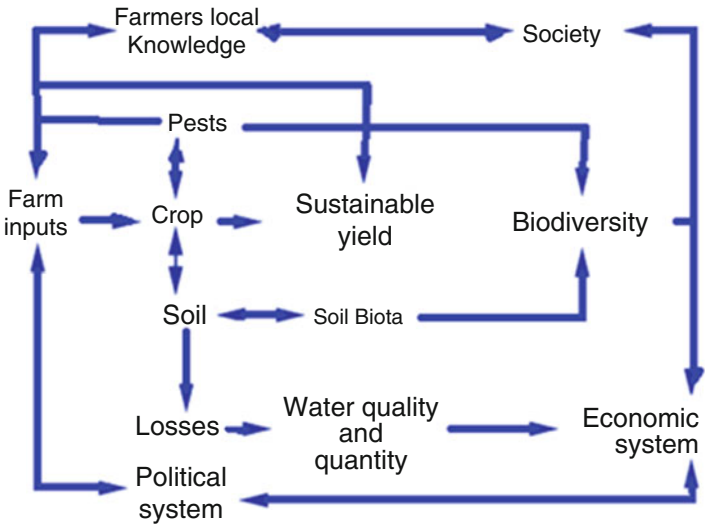

Fig. 1.1 Sustainable agriculture system

Sustainable agriculture is a model of social and economic organization based on an equitable and participatory vision of development which recognizes the environment and natural resources as the foundation of economic activity. It applies farming principles of ecology i.e. positive relationships between organisms and their environment. The term *sustainable agriculture* was first coined by the Australian agricultural scientist McClymont (1975). According to him, it is an integrated system of plant and animal production practices having a local/site-specific application for long term sustainability and has objectives to:

(a) Satisfy human food, fiber and shelter needs.
(b) Enhance environmental quality and the natural resource.
(c) Assure the most efficient use of non-renewable natural resources with integration of natural biological cycles.
(d) Strengthen the economic viability of farm operations.
(e) Enhance the quality of life of the entire society.

The M.S. Swaminathan Research Foundation (MSSRF) Chennai, India has developed an Integrated Intensive Farming System (IIFS) which is also known as ecological farming or intensification of natural resources. It involves agricultural intensification, diversification and value addition on a sustainable basis (Kesavan and Swaminathan 2008). This system is economically rewarding, intellectually challenging, energy efficient and employment oriented. It is based on intensive use of natural resources, traditional knowledge and the skill of farmers to increase productivity; efficient recycling of organic residues and a risk-minimizing integrated system. This system is self-reliant, transforming agriculture from chemical use to eco-farming, but its package is site or area specific (Fig. 1.2).

Achieving the goal of sustainable agriculture is the responsibility of all participants in the system: farmers, laborers, researchers, retailers, consumers and policymakers. Their cooperation and contribution will strengthen the sustainable agriculture system. Industrial agriculture consumes resources such as fossil fuel,

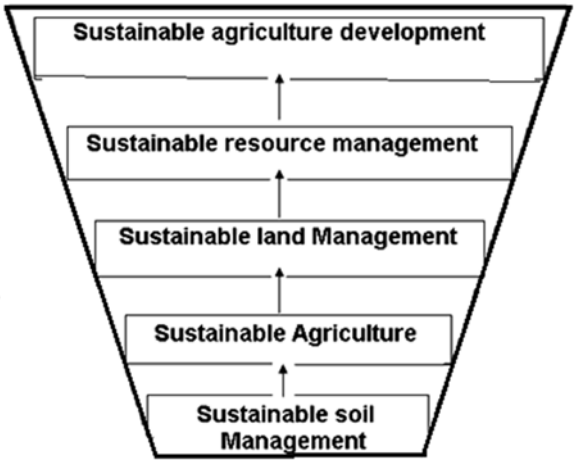

Fig. 1.2 Development of sustainability

Table 1.2 Difference between conventional and sustainable farming systems

No.	Conventional farming system	Sustainable farming system
1	Rely on technological innovation and skills	Rely on farmers knowledge and skill management
2	Management technology depends on high capital investment	Management technology depends on low capital investment
3	Large scale and big farms	Small and medium scale farms
4	Monoculture or single crop cultivation over many years	Diversified crop cultivation
5	Extensive use of fossil fuel fertilizers, insecticides and energy	Reduced use of fossil fuel, rely on alternate natural cycles of waste
6	Rely on mechanization and less labor	Rely on human resources and more labor demand
7	Rely on commodity supply chain	Rely on value addition and direct marketing
8	More transportation and high pollution	Less transportation and low pollution
9	Livestock depends on concentrated system	Livestock depends on pasture based system
10	High pollution to atmosphere and natural resources	Less pollution to atmosphere and natural resources

water, topsoil and contributes to degradation of natural resources such as air, water, soil and biodiversity. Therefore, judicious use of all natural and human resources is advocated and given prime importance for sustainable agriculture. The stewardship of land and natural resources involves maintenance and conservation of natural resources (Dobbs and Pretty 2004) for their long-term sustainability (Table 1.2). It is a way of producing food that is healthy to eat, does not harm the environment or animals, provides a fair wage to the farmer, and supports enhanced local community (Indicators for sustainable water resources development 2013). Sustainable agriculture includes the following objectives:

(a) Characterization, conservation, evaluation, and use of plant and microbial genetic resources of interest in the agro-alimentary sector.
(b) Use of genomic and proteomic tools to study heredity productiveness, adaptiveness and quality traits in plant breeding programs. The new plant cultivars should enhance efficiency, quality of products and growing sustainability.
(c) Interaction studies between soil, rootstocks, cultivar, auxiliary fauna, biological pest control and improvement of plant growth system.

1.3.1 Use of Decomposers for Sustainability

Sustainable agriculture should not deprive decomposers of biological waste. The living systems of the earth are capable of sequestering more greenhouse gasses than they release; there is need to learn and create sustainability to work with nature

rather than against it. Decomposers have enormous possibilities to produce a significant quantity of bio-energy while still allowing the decomposers to feed the plants. It is possible to become more efficient than it is today and to return sufficient agricultural organic wastes to the soil to be more beneficial to the decomposers.

1.3.2 Sustainable Resource Management

The increasing demand for food creates uncertainty because one of our greatest challenges is to increase food production in a sustainable manner with judicious use of natural resources, without over-exploiting them within balanced ecosystems (Cassman et al. 2002), so that everyone can be fed adequately and nutritiously. Sustainable farming must have a continuous flow of biological energy by recycling of nutrients through crop and livestock residues to the soil as organic matter restores healthy and productive organic soils that minimize the use of non-renewable inputs that are harmful to the environment, as well as the health of farmers and consumers (Goulding et al. 2008). Ecological agro-ecosystem management addresses energy-flows, the nutrient cycle, population-regulating mechanisms and system resilience. Sustainable agriculture outcomes can be a positive force for food productivity, it reduces pesticide use and carbon balances but it has significant challenges and needs the support of national and international policies of governance in developed and developing countries.

A better concept of resource management is centered on intensification of natural resources i.e. land, water, biodiversity (flora and fauna), technologies, genotypes and ecological management practices that minimize harm to the environment. The basic resources for agricultural development are prone to various forms of degradation. Therefore, it is necessary to develop strategies for conservation and improvement of these natural resources without compromising their stability for future use. The sun, air, water and soil are the four most important natural resources, of these, water and soil quality are highly subject to human intervention and pollution. Crop productivity is dependent on availability of soil nutrients and water, but without replenishment land suffers nutrient depletion and ultimately leads to reduced yields (Goulding et al. 2008). The authentic agricultural sustainability initiatives arise from shifts in the use of inputs of agricultural production e.g. from chemical fertilizers to nitrogen-fixing legumes; from pesticides to emphasis on natural biological control and from ploughing to zero-tillage. Sustainability of agricultural and resource management can be achieved by the following steps:

(a) Recycling of crops and livestock waste as manure.
(b) Growing legume crops to enhance bacterial symbioses to sequester atmospheric nitrogen.
(c) Enhance production of nitrogen fertilizer from natural gas.
(d) Use of renewable energy from hydrogen (water), solar and windmills.
(e) Use of genetically engineered (non-leguminous) crops to fix atmospheric nitrogen.

Water Water is the principal natural resource, without which agriculture is not possible; in future it may be a major limiting factor if mismanaged. An efficient use of water can establish high crop production. During the dry season groundwater is the only source to sustain the crops; therefore, it is necessary today to conserve ground water by adopting following practices:

(a) Recharge of ground water and its proper storage.
(b) Selection of drought-tolerant crop species/varieties.
(c) Using drip irrigation or sprinkler systems to encourage economic water consumption.
(d) Growing crops with efficient or minimum water use.

The quality of water can be conserved to reduce or stop salinization and contamination of surface and ground water from pesticides, nitrates and selenium. Salinization of soil becomes a major problem if the water table is near the root zone of the crops (Morison et al. 2008). The growing of salt-tolerant crops and drip irrigation can minimize the effects of salinization. Pesticide and nitrate contamination of water can be reduced by cultivating drought-tolerant forages, agroforestry and restoration of wildlife habitat on farms.

Wildlife Conservation of wildlife habitat on agricultural land can reduce soil erosion and sedimentation. The support of wildlife diversity will enhance natural ecosystems and agricultural pest management (Green et al. 2005).

Air Air pollution is mostly caused by smoke from the burning of agricultural waste, dust from tillage, harvest and transportation, spraying of insecticides, pesticide and emission of other harmful gasses like nitrous oxide from fertilizer. The burying of crops residue in the soil, planting wind breaks, cover crops and perennial grasses on open land can reduce air pollution.

Soil Healthy soil is one of the most important components of sustainability; it involves management of physical, chemical and biological properties to contain large quantity of organisms to maintain the soil ecosystem (Kibblewhite et al. 2008). Organic matter and compost are nutrients for beneficial soil micro-organisms such as protozoa, bacteria, fungi and nematodes. Properly managed soil organisms perform vital functions to grow healthy and more vigorous plants; therefore, they are less susceptible to pests. Regular additions of organic matter can increase soil fertility by increasing soil nutrients and microbes. Soil erosion caused by heavy storms, wind and flooding are a severe threat to crop productivity. The following methods can enhance the soil fertility.

(a) Crop rotation and cover crops. Rotation of two or more crops in the same field interrupts pest reproductive cycles, reducing the pest control practices and fertilizer requirements, as one crop provides nutrients for the subsequent crop (Huang et al. 2003). Planting of cover crops improves soil quality, prevents soil erosion, minimize weed growth and better economic gains (Sullivan 2011).

(b) Natural sources of soil nutrients. Indian hemp (*Crotalaria juncea*) and cowpea (*Vigna unguiculata*) can improve micronutrient availability; increasing soil organic content, physical properties, aggregate porosity, bulk density, water retention capacity and the fixing of atmospheric nitrogen (Sullivan 2004). Use of organic manures can improve soil health by reducing salinity, sodicity and alkalinity (Yadav et al. 2013). Use of forestry crops, ally crops, sericulture and wasteland reclamation also stops soil erosion. Agro-industrial waste, rice husk/bran, bagasse/press cake, cotton dust, oil cake, wastes from slaughter houses, waste from marine industry, crop residue, recycling of biomass, vermin-culture and vermin-composts, pro-fertilizers, i.e. *Rhizobium* and *Azotobacter*, are all examples. FYM (farm yard manure) viz. waste, excreta undigested protein, urea, Ca Mg, Fe, P. and slurry bleeding enhance soil fertility.
(c) No-till or low-till farming. No-till or low-till farming systems minimize disturbance to the topsoil residue cover and increases the retention of water and nutrients in the soil, making more water available for crops by improving infiltration and decreasing evaporation from the soil surface.

Farm Diversity Regional farm diversity can reduce the vulnerability of food production to climate change. Although monocultural farms are more advantageous in terms of efficiency and management, they are more vulnerable to pests and diseases (Fraser et al. 2005). Diversified farms are more resilient economically and ecologically and also create more niches for beneficial insects and is less risky to farmers. Annual cropping systems with crop rotation are more effective to suppress weeds, pathogens, insect pests, and to maintain stability of soil fertility and to promote soil moisture conservation.

Carbon Chain According to recent carbon footprint analyses, the entire chain of food production and consumption accounts for 20 % of global greenhouse gas emissions. Reducing these emissions and increasing the long-term storage of carbon in the soil are, therefore, essential measures to prevent climatic catastrophe (Lal 2008).

Sustainable Renewable Energy Sources Non-renewable energy sources will not be available indefinitely and their decline can be catastrophic in the future. Sustainable agricultural systems should not rely heavily on non-renewable energy sources and these must be replaced in the future with renewable, economical and feasible energy sources (Boyle 1996). Sustainable sources are biofuels, hydrogen, wind power, hydropower, geothermal energy, ocean energy, solar energy and bio-energy. Bio-energy from agriculture waste may lead to conserving food crops. Forage crops have more potential as crops for renewable biological energy because they capture about two-thirds of the total solar energy used in agriculture; together with forestry it can be doubled again. The use of forage crops to produce more renewable energy has two advantages: (a) most forage crops require less nitrogen fertilizer and pesticides than maize and soybeans, meaning less reliance on fossil energy in minimizing pollution and (b) most forage crops are perennial and require no annual tillage and thus cause less soil erosion; soil also being a good storehouse of biological energy.

Current technologies used to produce ethanol and biodiesel hold more promise to realize the limited use of non-renewable energy source and can replace petroleum energy significantly. Pyrolysis is another alternative for renewable energy source.

Pyrolysis This is another high-priority area of sustainable bio-energy and one of the most promising technologies of the day (Marker et al. 2012). Pyrolysis is a form of chemical decomposition of organic materials under high temperature in the absence of oxygen; this process has been extensively used in the chemical industry to produce charcoal, activated carbon and methanol from wood. The technology needs to be adapted more widely. Pyrolysis has several significant advantages over current methods of producing ethanol and biodiesel from maize and soybeans, as follows:

(a) Low-cost technology of varied sizes available for individual farmers, more feasible and cost competitive which can used for a wide variety of feed stocks (e.g. forages, non-food crops, wood and various wastes).
(b) A variety of useful products are produced such as biogas, bio-oil, chemicals, bio-char and tars.
(c) Bio-oil can be further converted into ethanol, biodiesel and biogases. Biogases can be again used to fuel the pyrolysis process to produce ethanol or biodiesel.
(d) Pyrolysis is less water consuming and does not pollute the air.
(e) Bio-char can be added back into croplands to promote soil fertility as organic matter that maintains soil ecology by promoting relationships between organisms and roots of the plants, water, carbon dioxide and nitrogen in the atmosphere.
(f) Bio-energy crops used in pyrolysis may sequester more greenhouse gasses than are released, as part of carbon is retained in the soil by the plant roots.

Further it is possible to find ways to generate significant quantities of bio-energy from the flow of the earth's biological pyramid and nutrient recycling processes within ecological limits of sustainability, without compromising the efficiency and integrity. However, the truly renewable energy are solar energy and hydropower.

1.4 Strengthening of Natural Agro-Ecosystems

Agricultural sustainability emphasizes the potential benefits from improved genotypes of crops and livestock and their agro-ecological management. It includes genetically modified organic crops; provided they improve biological and economic productivity for farmers and do not harm the environment, along with ecological conditions under which they are grown. Higher productivity output is a result of interaction between genotype and environment (GxE) (Khush et al. 1988). Agricultural sustainability focuses on both genotype improvements through modern biological approaches, as well as improved understanding of ecological and agronomic management, manipulation and redesign (Collard and Mackill 2008; Flint and Wooliams 2008; Thomson 2008).

Mature ecosystems must be in a state of dynamic equilibrium that acts as a buffer against large shocks and stresses. The present agro-ecosystems have weak resilience for transitions towards sustainability that need to focus on structures and functions that may improve resilience (Folke 2006; Holling et al. 1998; Shennan 2008). Sustainable agro-ecosystems have to balance the plant properties towards the natural ecosystem without reducing productivity. To improve natural agro-ecosystem, biological diversity needs to create increased natural control and regulatory mechanisms to manage biotic factors (pests and diseases) rather than to eliminate them from the ecosystem. The conversion of traditional to sustainable agro-ecosystem is a complex process requiring a site-specific approach to restore resource management which is contributed by a number of important factors, their interactions and interrelationships; these factors include:

(a) Integrated Pests Management (IPM). This technique uses natural/biological ecosystem resilience, diversity of pest, disease and weed control; chemical pesticides are used only as a last resort when other options are ineffective (Bale et al. 2008; Hassanali et al. 2008). To keep destructive insects under control IPM emphasizes crop rotation, intercropping and other methods to disrupt pest life cycles. The cultivation of resistant Bt (*Bacillus thuringiensis*) plant varieties is also recommended to control pests.
(b) Integrated Nutrient Management. This includes balancing biological nitrogen fixation within farming systems and supplementing with inorganic and organic sources of nutrients to soil if needed (Goulding et al. 2008).
(c) Agroforestry. This approach includes planting multifunctional trees in agricultural systems and their collective management to develop as nearby forest resources.
(d) Aquaculture. This involves development of aquatic fauna such as fish, shrimp and other aquatic resources into irrigated rice fields and fish ponds which enhances the protein production in farming systems (Bunting 2007).
(e) Water Harvesting. Owing to better rainwater retention in dry land or degraded land areas, crops can be grown on small plots under irrigation to improve water harvesting (FAO 2013; Morison et al. 2008; Pretty 1995; Reij et al. 1996).
(f) Livestock Integration and Rotational Grazing. In farming systems this consists of keeping livestock viz. dairy cattle, pigs and poultry, including using zero-grazing, and cut and carry systems (Altieri 1995; Wilkins 2008) which can enhance farm sustainability. Rotational grazing and periodic shifting of animals to different areas, prevents soil erosion by maintaining sufficient vegetative cover, it also saves on animal feed costs, distributes the manure and contributes to soil fertility. The implication and adoption of the abovementioned practices can make several favorable component changes in the farming system. The growing of hedge rows and alley crops encourages many pest predators and also act as wind breaks to reduce soil erosion. The rotation of legume crops helps to fix atmospheric nitrogen and also acts as a barrier crop to prevent carry-over of pests and diseases. Grass contour strips slow surface-water run-off which encourages percolation to recharge the water table and can be used as fodder for

livestock. Catch cropping prevents soil erosion and leaching during critical periods and is a source of green manure which may increase soil organic matter with water-retention capacity.

(g) On-farm Biological Processes. These processes include the rebuilding of depleted natural buffers of predator stock and wild host plants that increase the levels of nutrients, enhancing exploitation of micro-environments and positive interactions between them which promotes sustainable productive agro-ecosystems (Firbank et al. 2008; Kibblewhite et al. 2008; Wade et al. 2008).

(h) Efficient use of Inputs. It has been observed that sustainable approaches are less toxic, less energy intensive and more productive and profitable to farmers. This maximizes reliance on natural renewable on-farm inputs which largely impacts on the environmental, social and economic strategy. Conversion of any conventional farming system to a sustainable system does not mean replacement of chemical inputs only, but it substitutes enhanced resource management and use of scientific knowledge of packages and practices on farms and in rural communities.

(i) Zero Waste Agriculture. This approach represents a step towards sustainable agriculture which optimizes the use of five natural kingdoms, i.e. plants, animals, bacteria, fungi and algae to produce biologically diverse food, energy and nutrients in a synergistic integrated cycle and profit-making processes where the waste of one process becomes the feedstock for another.

(j) Urban Agriculture. World urbanization is increasing at a rapid rate which is reducing the amount of agricultural land and its production. But this urbanization can also be used for human welfare if urban agriculture becomes an important component for agricultural sustainability. Urban agriculture can include animal husbandry, aquaculture, agroforestry and horticulture. As production is close to the consumer, it can reduce transportation costs, pollution from storage and packaging cost. Urban agriculture will also help in the disposal of urban waste, create economic development and improve food security in poor communities (Butler and Moronek 2002).

(k) Participatory Plant Breeding. The Green Revolution has left millions of farmers in developing countries in an uncertain situation; most of them operate small farms under unstable and difficult growing conditions. Adoption of new plant varieties by these farmers has been hampered by their lack of financial resources and existing policies of proprietary rights which have been implemented to promote an industrialized model of agriculture. The participatory plant breeding technology and the involvement of farmers in new research on varieties can be a good option to insure their participation (Pixley et al. 2007). In many countries farmers and scientists have begun participatory plant breeding which is the best way to breed for sustainable agriculture; a good example is the organic durum wheat program in France.

1.5 Plant Breeding Methodology for Sustainable Agriculture

Classical plant breeding programs have been very successful in producing cultivars suitable for favorable environments, together with necessary excessive use of fertilizer and chemicals for control of weeds, pests and diseases, which have increased agricultural production several fold. The current high-input agriculture system has more unfavorable environmental impacts and is causing increasing concern with crops. The contribution of plant breeding to sustainable agriculture is important and based on following objectives:

(a) Enrichment of the source material with landraces and cultivars, accompanied by appropriate breeding methods for yield enhancements.
(b) Screening of cultivars, breeding techniques and their genotypic profiling.
(c) Selection in segregating generations should be based on individual plant evaluation and performance which can reduce genotype × environment (G×E) interaction with increased heritability.

These abovementioned techniques and further selection criteria can maximize heritability gain and provide efficient solution in resolving sustainable agricultural problems.

1.5.1 Sustainable Use of Plant Genetic Resources

Plant genetic resources remain a key component of global food security, and to assure future peace and prosperity. Plants are the major source for human sustenance, directly for human food, clothing and shelter, and indirectly in processed commodities and as animal feed. Since ancient times, crop plants have been subjected to several evolutionary forces and human selection processes from their wild ancestors to the domesticated/cultivated forms of today. The genetic diversity expresses individual traits, representing variation in the molecular building blocks of DNA which are at the core of a crop's ability to undergo continuous variations. The combination of current breeding technology and genetic diversity are of great potential to adapt crops according to the choices of farmers and consumers. Plant genetic resources in agriculture have undergone evolutionary processes of conservation, diversification, adaptation, improvement and then seed production systems. As genetic resources, traditional varieties are conserved in gene banks; in this regard the first gene bank was created in the 1930s. There are now around 1,400 gene banks functioning worldwide, maintaining more than 504 million accessions. In situ and ex situ conservation gene banks are also maintained by farmers but without conservation information/data this biodiversity resource is underutilized for breeding purpose (Cohen et al. 1991). Plant genetic resources are the backbone of agriculture and play a positive and unique role in the development of new cultivars (Malik and Singh 2006). Plant breeding functions as a bridge between the

conservation in gene banks and production of improved varieties and their distribution among farmers for cultivation. For sustainable use of genetic resources, FAO is playing a key role under the following two strategies: (a) appropriate policies and strategies at the regional, national and international levels to create a favorable environment for sustainable use of plant breeding and seed sectors and (b) capacity building, providing educational and training support in developing countries through various institutions in plant breeding and the seed sectors.

1.5.2 Agriculture Biodiversity and Food Security

Agricultural biodiversity is the foundation of food security throughout the world; species biodiversity and farming systems provide valuable ecosystem functions for agricultural production (FAO 1996). The sustainable use of conservation and enhancement of agro-biodiversity is valuable for insuring food security because there are several serious threats to loss of agro-biodiversity; therefore there is a critical need to rapidly adopt an agro-ecosystems approach to genetic resource conservation and integrated ecological pest and soil management.

1.5.3 Plant Genetic Resources

Diverse genetic resources provide an array of potentially useful traits to the plant breeder which are exhibited in landraces and wild varieties, for resistance to diseases, pests and environmental stresses (e.g. heat, drought, cold) that are highly desirable in achieving improvements in crops (Brown et al. 1989). In this context, a good example is the rice grassy stunt virus disease that seriously damaged rice fields from Indonesia to India in the 1970s. About 6,273 varieties were tested for resistance to the virus; ultimately an Indian variety was found suitable to breed successful resistant hybrid varieties which are still widely cultivated. Similarly, in other countries, rich biodiversity exists in crops such as cereals, fruits, vegetables, industrial crops, oil crops and forages, which can contribute significantly to agricultural diversification (Feehan et al. 2005). By recombining this genetic diversity, many new varieties have been developed by modern plant breeders with improved traits never before available to farmers.

1.5.4 Exploitation of Existing Germplasm

Only a scant 0.1 % of the world's plant species are grown as crops and only a very small proportion of the total genetic variability is represented by commercial varieties. Exploitation of plant diversity can be used to achieve breeding objectives in

major crops, to develop new crops, to meet emerging needs from existing crops, to ensure benefit to the world's population from breeding programs and to ensure the sustainability of crop production. Species that are rarely cultivated and genes from accessions and species related to existing crops, can be exploited to satisfy the need for improvement of agricultural production. Molecular and statistical methods have the potential to speed up introduction of novel germplasm, to allow quantitative assessment of diversity, characterization of desirable genes, tracking of chromosomes, genes, or gene combinations through breeding programs, selection of rare recombination and direct gene transfer through transformation. Several underutilized plants have potential for improving agriculture in diversified agro-ecological niches; these have great potential for exploitation as economic products for food, fodder, medicine, energy and industrial purposes (Bhag Mal and Joshi 1991).

1.5.5 Breeding for Abiotic Stresses

Agricultural sustainability depends on genotypic improvements and its environmental interaction through modern biological approaches and better understanding of ecological, agronomic management, manipulation and redesigning or remodeling of crops. As in the case of cereal crops, abiotic stress factors such as low nitrogen, drought, salinity and aluminum toxicity causes large and widespread yield reductions; therefore, breeding for tolerance to these abiotic stresses is important (Bänziger et al. 1999). Drought severity and occurrence is predicted to increase with climate change and the area of irrigated land is subject to increased salinization and the cost of inorganic N is set to increase. Breeding for low N can be adopted while retaining the ability of the crop to respond at high N input. Breeding for drought and salinity tolerance together is a difficult and complex trait but this mechanism can be explored by marker-assisted selection (MAS) for component traits. MAS for drought tolerance in rice and pearl millet, and salinity tolerance in wheat, has evidenced some encouraging results by the pyramiding of stable quantitative trait loci which is controlling the component traits. The understanding of processes and identification of responsible genes for genomic technologies has revealed great potential for breeding tolerance to drought and salinity stresses in wheat due to contributions from their wild relatives.

1.5.6 Wide Hybridization

Plant tissue culture techniques have successfully produced progenies in a number of useful crosses in distantly-related species. Several interspecific and intergeneric hybrids have been produced by crossing related species/genera that do not normally reproduce sexually; these crosses are referred to as *wild crosses*, as in the case of triticale which is a hybrid between wheat and rye, where the F_1 progeny was sterile

Fig. 1.3 Wide hybridization between, wheat and rye produced triticale grain

due to an uneven number of chromosomes but by doubling of the chromosomes (with colchicine) during cell division which inhibited cytokinesis, a fertile line was produced by Rimpu in 1890 (http://www.agriinfo.in/). This sterility in the F_1 hybrid may be due to pre- or post-fertilization incompatibility.

If fertilization occurs between two species or genera of wheat and rye (Fig. 1.3), the hybrid embryo may abort before maturation; if this happens the embryo can be rescued by an embryo rescue procedure and cultured to produce a whole plant. Using this technique an interspecific cross of Asian rice (*Oryza sativa*) and African rice (*O. glaberrima*) has produced a new rice for Africa. Hybrids can also be produced by protoplast fusion techniques where protoplasts are fused together, usually in an electric field and viable recombinants regenerated in culture media.

1.6 Organic Agriculture

The objective of sustainable agriculture is to encourage crop production with reduced use of non-renewable inputs such as nutrients, pesticides, water and other maintenance; this concept was formulated by Albert Howard, an English botanist and organic farming pioneer, in the 1940s. In recent years the use of natural resources, management of plant diseases, insects and pests, has begun to point the way for sustainable agricultural plants as invaluable resources. Careful plant selection is the first important step in developing a balanced and self-perpetuating farming system. Plant survival with minimal maintenance is not the only issue in sustainability for there is a difficulty with invasive exotic plants, displacing native plants and disrupting natural ecosystems. Sustainable agriculture mainly is a rejection of the industrialized food production and gear-up to feed the ever-growing global human population. Organic agriculture seems to be the best approach for sustainable agriculture because it is a rapidly growing sector that focuses on

agricultural health, ecology, justice and care of the farming process that utilizes local resources and offers opportunities for increasing farm income and to feed the world sustainably in the future (Lampkin and Padel 1994). Organic farming can also contribute to the goals of sustainable agriculture in the following ways:

(a) Water use efficiency.
(b) Enhance soil water retaining capacity.
(c) Nutrient use efficiency.
(d) Competitiveness of crops over weeds.
(e) Tolerance to mechanical weed control.
(f) Early maturity to avoid biotic and abiotic stresses.
(g) Crop rotations and use of organic waste manures to maintain soil fertility with high humus.
(h) Biological insect pest and disease management.
(i) High root growth development.

1.7 Contribution of Genetics and Plant Breeding to Sustainable Agriculture

Genomic tools have provided a better understanding of structure and analysis of the plant genome for development and genetic controls that stimulate cascades of gene expression at various life-cycle stages that facilitate the stages of morphogenesis of vegetative, reproductive organs (i.e. germination to reproduction and seed production). The new technologies enable the breeder to identify the gene activity and responses to the biotic and abiotic challenges faced by the plants. The increased knowledge and powerful gene sequence-based diagnostics have provided plant breeders with more precise selection objectives from a large breeding population of new modified varieties by phenotypic observations (Dennis et al. 2008). By using some of these genetic tools, achievements have been made in the agriculture sector beyond the food, feed and fiber industries which have contributed high-value products in agriculture and the pharmaceutical industry. Some of these tools are given below:

(a) Backcross Breeding (BC). The cross between F_1 progeny with either of the parental genotype; the resultant progeny is called BC_1 and the progeny of the cross between BC_1 and the recurrent parent is known as BC_2.
(b) Gene Pyramiding. The process of aggregation/accumulation of the favorable gens/alleles from different genotype sources into elite/commercial cultivars is generally performed through marker-assisted selection (MAS).
(c) Linkage Disequilibrium (LD). The non-random association of alleles at different loci, describing the condition with non-equal (increased or decreased) frequency of the haplotypes in a population at random combinations of alleles. Linkage disequilibrium is different from the linkage, although tight linkage may generate high levels of LD between alleles.

(d) Narrow Genetic Base (NGB). This exists frequently in modern crop cultivars or breeding lines due to the continuous use of a small number of elite genotypes in a breeding program. It is a serious obstacle to sustain and improve crop productivity due to rapid vulnerability of genetically uniform cultivars to emerging biotic and abiotic stress in crop plants.
(e) Next Generation Sequencing (NGS) Technologies. NGS includes various novel sequencing technologies like 454/FLX (Roche Inc.), ABI SOLiD and Solexa that have surpassed traditional Sanger sequencing in throughput, which is cost-effective in generating large-scale sequencing data.
(f) Polygenes. Polygenes are groups of non-allelic genes, having small quantitative cumulative effects which produce a wide range of phenotypic variations.
(g) Quantitative Trait Loci (QTLs). These are loci in the genome that contribute towards conferring tolerance to abiotic stresses (drought and salinity), resistance to biotic stresses (fungal, bacterial and viral diseases) or improving agronomic traits (yield and quality); those are generally controlled by polygenes and greatly influenced by interactions between genes and environment.

1.8 Role of Plant Biotechnology and Genetic Engineering

The era of plant biotechnology began in the 1980s with transgenic plant production using *Agrobacterium* (Bevan et al. 1983; Fraley et al. 1983). The successful commercialization of transgenic crops and the integration of biotechnology into plant breeding and crop improvement programs was accomplished by Koziel et al. (1993) and Delannay et al. (1995). At present, introgression of one or a few genes into an elite cultivar through backcrossing is a frequent practice in plant breeding. Biotechnology can be used to meet the growing global demand for food by improving nutritional quality of crops and reducing impact on the environment (USDA 2005). Through the advancements of biotechnology, GM (genetically modified crops) have contributed substantially to sustainable agriculture in developed countries for food and other commodities. Now there is urgent need to extend these GM crop developments into developing countries. FAO suggests that biotechnology has enormous possibilities to solve problems of hunger, food security and nutritional improvement. Adoption of biotechnology will contribute to solving the problem of climate change and also enhance its global impact to advance scientific knowledge and provide economic benefits to developing countries (e.g. Ghana, Nigeria, South Africa, Swaziland and Kenya).

The achievements of plant biotechnology have surpassed all previous expectations and its future seems bright and more promising. The intensification of agriculture requires enhanced and efficient plant breeding techniques in the release of economical, high yielding and patentable plant-derived products. This can be achieved with continuous support of advanced breeding research techniques and development of crops in biochemistry, physiology, biotechnology genomics, proteomics, transcriptomics and metabolomics studies. Biotechnology enabled access

to genes that are not possible through crossing and opened an infinite pool of novel genetic variations. Genes can be acquired from any existing organism genome, designed and assembled de novo in the laboratory as per requirements, as transgenes for resistance to glyphosate herbicides in maize and soybean.

Plant breeding and biotechnology can help in food production but it does not have all the solutions. Improved crop varieties can be incremental rather than transformative. Seasonal food should be appreciated and the need to make better use of the food produced. Due to higher meat costs, many people will shift towards a more vegetarian diet, provided by farmers growing diverse crops in local communities and regions. This situation may raise the possibility that developing nations might be better adapted to produce food under sustainable conditions with low inputs as compared to Western nations that are currently hooked on high intensity agriculture. Thus continued economic growth can be the biggest threat; there is also the ultimate need to campaign for controlled human population and less dependence on consumption of non-renewal energy and natural resource management.

1.9 Precision Breeding for Future Agriculture

Molecular markers and other genomic applications have been highly successful in characterizing existing genetic variations within species. Further advances have led to methods of maker-assisted backcrossing for the introgression of transgenic traits and linkage drag. Since then the continuous development in the application of plant biotechnology, molecular markers and genomics has established new tools for the creation, analysis and manipulation of genetic variation in the development of improved cultivars (Collard and Mackill 2008; Sharma et al. 2002). At present molecular breeding has become a standard and indispensable practice of crop breeding. Plant biotechnology generates new genetic diversity that often extends beyond species boundaries (Gepts 2002; Johnson and McCuddin 2008). Conventional plant breeding has a major limitation in that only closely-related species can be crossed and resultant qualities cannot be predicted because hundreds of genes are being moved around. Using genetic engineering tools to cut and paste DNA to create new genes gives results that depend on a specific gene combination and its transfer across species and beyond, has created new possibilities (Bongiovanni and Lowenberg-Deboer 2004). Thus, genes can be transferred, for example, from plants to animals, animals to plants and bacteria to plants. The development of recombinant DNA (rDNA) technology is credited to American scientists Paul Berg, Herbert Boyer and Stanley Cohen, who provided the tools to understand the functions of specific genes and led to the field of molecular biology that is now revolutionizing biology. Recombinant DNA technology provided the tools to transfer a specific gene to achieve desired characteristics with precision and therefore more predictable outcome become possible; the techniques is called *precision plant breeding*. This technology is being deployed in food crops such as maize, wheat, rice, and to non-food crops in tailoring existing crops to face new challenges, such

as climate change, incorporating valuable traits from wild relatives into established crops. The progress in precision plant breeding can help to meet the new challenges with the following objectives:

(a) Shorten the time to domesticate new crops from semi-wild plants.
(b) Tailor existing crops with traits like nutritional enhancement or climate change.
(c) Rapidly incorporate valuable traits from wild relatives into established crops.
(d) Facilitate plant breeders to work with complex traits such as hybrid vigor and flowering.
(e) Makes possible research to work on neglected or *orphan* crops.

In recent years plant genomics technologies have contributed greatly in agriculture and have led to better understanding of how plant genes function, their response to environment in performance and productivity improvement of the crops. The targeted objectives can be easily achieved in breeding programs with the help of molecular tools in characterization, conservation and better use of genetic resources. Recent advances in molecular tools have provided considerable opportunities in enhancing the effectiveness of bioinformatics and classical plant breeding programs, so the success of breeding programs can be effectively analyzed for large numbers of crosses at early seedling stages by genomics-assisted breeding. In this approach both the phenotype and the genotype of new varieties can be analyzed and performance of new introgressed traits can be predicted during the early selection stage.

Marker-assisted breeding has enormous potential to improve conventional plant breeding and make it more efficient and accurate. The large number of quantitative trait loci (QTLs) mapping studies for diverse crop species have provided an abundance of DNA marker-trait associations. The applications of MAS (marker assisted selection) are now widely used in breeding of several cereal crops (Fig. 1.4). The greater adoption of MAS is expected in the near future and the extent will depend on available resources. MAS has created the foundation for molecular plant breeding, an interdisciplinary science that is revolutionizing twenty-first century crop improvement. Precision breeding depends on advances in understanding of plant biology, analysis of genetic variations, cytogenetics, quantitative genetics, molecular biology, biotechnology and genomics in their application to the plant breeding process (Baenziger et al. 2006; Jauhar 2006a, b; Varshney et al. 2006). Molecular marker systems began in crops with high resolution genetic map creation and thereafter exploitation of genetic linkage between markers and important crop traits began (Edwards et al. 1987; Paterson et al. 1988).

In plant breeding several different genes often influence a desirable trait. The use of molecular markers/DNA fingerprinting can map thousands of genes; this facilitates plant breeders to screen and identify a large population of plants for a trait of interest in the laboratory phenotypically (Fig. 1.5). This is a process of indirect selection for improving the traits of interest by using morphological, biochemical and DNA based/molecular markers which have proved to be a good choice in the breeding program of MAS, as discussed below:

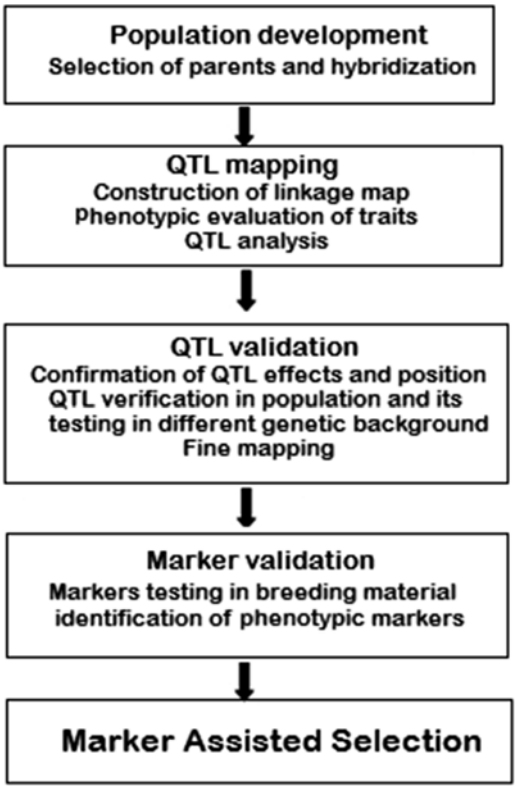

Fig. 1.4 Marker assisted selection

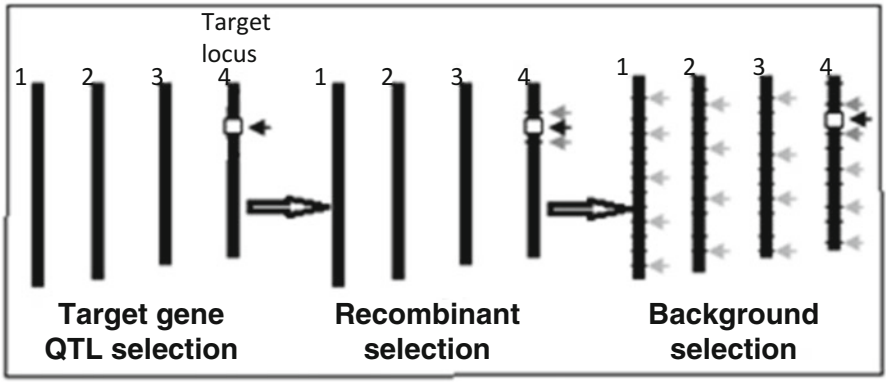

Fig. 1.5 Molecular marker assisted backcrossing and selection

(a) Association Mapping. A high-resolution mapping method to analyze and detect complex genetic traits for gene(s) quantitative trait loci (QTLs) for traits of interest based on linkage disequilibrium (LD).

(b) Genome Selection (GS) or Genomic Wide Selection (GWS). A breeding strategy for accelerating genetic gain especially for complex traits in elite genotypes by utilizing genomic information and estimating their breeding values. Genomic selection is becoming more popular over marker-assisted selection due to its advantages and the recent advances in genomic technologies that have drastically reduced the cost of marker genotyping (Heffner et al. 2009).

(c) Genomics-Assisted Breeding (GAB). This approach uses technologies such as molecular markers, transcriptomics, metabolomics, proteomics, bioinformatics and phenomics, which are integrated with conventional plant breeding strategies for three traits in crop plants: resistant (or tolerance to biotic and abiotic stresses); improved quality; yield enhancement.

(d) Gene Pyramiding. Pyramiding is the process of combining several genes together into a single genotype which may also be possible through conventional breeding, but it is usually difficult to identify plants containing more than one gene. DNA markers can greatly facilitate selection because DNA marker assays are non-destructive and markers for multiple specific genes can be tested using a single DNA sample without phenotyping. The most widespread application for pyramiding has been for combining multiple disease resistance genes such as combining qualitative resistance genes together into a single genotype (Kloppers and Pretorius 1997; Shanti et al. 2001; Singh et al. 2001). Finally, the availability of large numbers of publicly-available markers and the parallel development of user-friendly databases for the storage of marker and QTL data will undoubtedly encourage the more widespread use of MAS. Advances in functional genomics will lead to the rapid identification of gene functions in the major cereal crops. This strategy usually relies on fine mapping using molecular markers, as well as other methods such as gene-expression studies (microarray), mutants and gene knockouts, RNAi and association genetics. The identification of gene function will allow the development of allele-specific markers that will be more efficient than using linked DNA markers to find more useful alleles.

(e) Molecular Plant Breeding and Genetic Gain. The maximum potential for genetic gain is proportional to the phenotypic variation (бp) present in the source population and maintained in subsequent cycles of selection. Phenotypic variation is positively associated with genetic diversity, and depends on environmental factors and the interactions between genotype and environment (GxE). Genetic diversity may be derived from breeding populations, segregating progeny of a cross of selected parental lines. All phenotypic variations are not equal, as exotic germplasm has been extremely successful for improving many crop species, but difficulties are encountered through the introduction of undesirable alleles associated with lack of adaptation. The need for genetic diversity must be balanced by elite performance, choosing the best parent is an input in maximizing the probability for successful improvement.

(f) Molecular Plant Breeding and Favorable Gene Action. Quantitative genetics uses the theoretical concept of heritability to quantify the proportion of phenotypic variation that is controlled by genotype. But heritability is greatly

influenced by the genetic architecture of the trait of interest, which is governed by the polygenes, the magnitude of their effects and the type of gene action associated with phenotypes. A better understanding of genetic architecture and favorable gene action which is more amenable to selection often has the greatest impact on improvement in genetic gain. For the genetic gain heritability (h^2) it is used in its narrow sense, representing the proportion of phenotypic variation due to additive genetic effects or allelic substitutions that are referred to as the breeding value because they are transmitted as expected to progeny. Deviations from additive effects are significant for many traits, and are partitioned into either dominance effects that reflect the interactions between different alleles at the same locus or epistatic effects resulting from interactions among different loci. Gene action and breeding values are measured by progeny testing, where the phenotypes of individuals in a population are compared to their parents and siblings produced from either self-pollination or out-crossing. The large number of molecular markers, high density genetic maps, and mapping populations define gene action and breeding value at hundreds or thousands of loci distributed across the entire genomes. The genomic segments can be readily identified that shows statistically significant associations with quantitative traits loci (QTLs). Either genetic linkage or epistasis among loci with antagonistic effects on a trait limits genetic gain, QTL information can be used to break these undesirable allelic linkage interactions. Molecular plant breeding increases favorable gene action and QTL studies identify causal genes that represents a powerful functional genomics approach. The molecular cloning of QTLs has yielded novel insights about quantitative traits that were not likely to be exposed from the analysis of gene knockouts or over-expression strategies. Molecular markers, genomics and biotechnology are now applied to exploit genetic diversity for crop improvement. Genomic information enables breeders to discover beneficial alleles using QTL mapping and cloning, followed by the molecular characterization of QTLs to design optimal transgenic strategies for sustainable crop improvement.

(g) Molecular Plant Breeding and Selection Efficiency. Conventional plant breeding relies on phenotypic selection with the highest breeding values, but it is expensive and time consuming. Molecular plant breeding helps to expand genetic diversity, characterizing genetic architecture and modifying gene action, its methods can be applied to increase the efficiency of selection. If genetic correlations are high, further efficiencies can be achieved by substituting genotypic with phenotypic selection during the selection process. Thus, molecular markers can be used to increase the probability of identifying truly superior genotypes. Marker-assisted selection also accelerates the exploitation of transgenes in commercial cultivars by marker-assisted backcrossing (Collard et al. 2008). However, to find future genetic improvements for tolerance to drought or nutrient deficiency, future breeding may be required to recognize transgenic expression because endogenous genes and environmental factors may have the potential to influence the phenotypes arising from transgenic modifications (Mumm 2007). The area of biotechnology, DNA marker technology, derived

from research in molecular genetics and genomics, offers great promise for plant breeding. Owing to genetic linkage, DNA markers can be used to detect the presence of allelic variation in the genes underlying these traits. By using DNA markers to assist in plant breeding, efficiency and precision could be greatly increased.

(h) RNA Interference (RNAi). Genetic modification in plants is achieved by adding a specific gene/genes or by knocking down a gene with RNA interference (RNAi) to produce a desirable phenotype such as increased shelf life; for example, the Flavr Savr tomato. Genetic modifications can produce a plant with the desired trait or traits faster than classical breeding because the majority of the plant's genome is not altered.

(i) Insect Resistance. Commercially released transgenic plants that have introduced resistance to insect pests and herbicides are still very limited. Insect resistance has been achieved through incorporation of a gene from the bacteria *Bacillus thuringiensis* (Bt) that encodes a toxic protein for some insects, as in case of Bt cotton, the pink bollworm a common cotton pest if feeds on Bt cotton it will ingest the toxin and die.

(j) Herbicide Tolerance. Herbicide tolerance usually functions by binding to certain plant enzymes and inhibiting their action. The enzyme that inhibits the herbicide is known as the herbicides *target site*. Herbicide resistance can be incorporated into crops by expressing a target site protein that is not inhibited by the herbicides; using this technology, glyphosate resistant crop plants have been produced in soybean.

(k) Reverse Breeding and Doubled Haploids (DH). A method for efficient production of homozygous plants from a heterozygous plant that has all the desirable traits, this can induce production of doubled haploid from haploid cell, and later create homozygous/doubled haploid plants from these cells. In natural offspring genetic recombination occurs and traits can be unlinked from each other, in doubled haploid cells and as a result DH plants recombination can be obtained. There is a recombination between two corresponding chromosomes that stay linked on alleles or traits; this leads to recombination between its identical copies. Therefore, traits on one chromosome stay linked, selection of offspring having the desired set of chromosomes and crossing results in a F_1 hybrid plant that has exactly the same set of chromosomes, genes and traits as in the heterozygous hybrid plant. The homozygous parental lines can reconstitute the original heterozygous plant by recombination if needed, in a desired quantity. A heterozygous plant can be converted into a heterozygous variety (F_1 hybrid) without vegetative propagation as a result of the cross of two homozygous/doubled haploid lines derived from the initially selected plant. Some successful results were achieved in bell pepper, fruit crops (e.g. citrus, banana and grapes), rapeseed, barley, wheat and rice and other cereals.

(l) Genotyping and Phenotyping. Utilization of the potential of each genotype needs the data of each phenotype which can be interpreted into a powerful

tool in improving yields in less favorable environments (Ceccarelli 1996). The contribution of plant breeding to sustainability is to: decrease GxE interactions and increase heritability through continuous selection and cultivar evaluation. The approach of GxE interactions provides tools for separating genetic effects from environmental effects, i.e., to get maximum yield and stability in macroenvironments (Ceccarelli 1989). Therefore, it is the extent of performance of any genotype given under different environments.

1.10 Mitigate Climate Change

An increase of temperature and carbon dioxide concentration will impact agriculture in different ways. Temperature increase can make many crops grow faster, but will also reduce yields. In case of some cereal crops, faster growth reduces the maturity period but produces small and immature grains. This effects of increased temperature will depend on the crop's optimum temperature required for growth and reproduction (USGCRP 2009). The climate change may effect agriculture in following ways:

(a) Higher CO_2 levels can increase yields in some crops like wheat and soybeans to 30 % or more under a doubling of CO_2 concentrations but maize can exhibit a smaller response i.e.<10 % increase (Backlund et al. 2008). The higher level of increase in temperature, CO_2, precipitation, floods and droughts can further harm or prevent crops from growing or to grow with reduced yields.
(b) Numbers of weeds, pests and fungi thrive under warmer temperatures, wetter climates, and increased CO_2 levels.
(c) In general, benefits will be small and outweighed by the negative impacts of higher temperatures, water limitations and extreme weather events (Ainsworth and Ort 2010; Long and Ort 2010). Sporadic heat waves have serious effects on yields such as the temperature increase of +6 °C in Europe in the summer of 2003 which caused record crop losses. Fertility and grain filling are adversely affected by high temperatures.

Global warming can result in the shift of crop producing areas towards the poles; the wheat belt of North America is predicted to shift northwards into Canada as temperatures rises and yield may drop due to poor soil quality farther north. In Australia the wheat belt is moving southwards but is limited by the southern ocean. Eurasia will face a similar migration, shifting production from one country to its northern neighbors. Other threats include changes in the distribution and severity of plant pests, disease, rising sea levels, flooding, storms, decline in soil quality (e.g. erosion, salinity) and diversion of resources into growing energy crops for biofuels rather than food crops. This type of industrialization of agriculture will create adverse conditions for food production strategies.

1.11 Breeding Better Future Crops

In the context of current yield trends, predicted population growth and pressure on the environment, traits relating to yield stability and sustainability should be a major focus of plant breeding efforts. These traits include durable disease resistance, abiotic stress tolerance and nutrient and water-use efficiency (Slafer et al. 2005; Trethowan et al. 2005). Furthermore, there is a need to develop varieties for cultivation on marginal lands, especially in developing countries, and to give greater emphasis to improving minor or orphan crops (Naylor et al. 2004).

As discussed earlier in this chapter the challenges ahead by 2050 of increased population will demand enhanced food, clothing, shelter, animal feed and fuel demand from agricultural products. The primary challenge is to expand agricultural output without significantly increasing environmental hazards due to maximum use of non-renewable inputs, irresponsible deforestation to clear land for farming, housing and urbanization. Therefore, it is necessary to find new ways to grow vertically by increasing crop yields. The major challenge is to increase agricultural yields by reducing the use of fossil fuels, water, and other negative input impacts on environment by considering the integral role of modifying genes utilizing all breeding techniques from pre-Mendelian times until today. Novel gene combinations can be achieved by spontaneous or induced mutations in the wild forms or by jumping genes sequences from one part of a plant genome to another, using biotechnology tools which can result in spectacular yield increases.

1.11.1 Salt-Tolerant Wheat

Soil surface salinity due to rising water tables, and natural or subsoil salinity, impose significant constraints on crop yields. The Commonwealth Scientific and Industrial Research Organization (CSIRO) in collaboration with wheat breeding companies in Australia is involved in breeding of salt-tolerant wheat varieties. This will provide the opportunity to farmers in salt-affected areas to increase the yields of both bread and durum wheat.

1.11.2 High Rainfall Zone Wheats

CSIRO is developing high-yielding, disease-resistant, milling-quality wheat varieties tailored to Australia's high rainfall zones. CSIRO has released numerous dual-purpose feed wheats which are suitable for grazing by sheep or cattle as well

Fig. 1.6 Cultivation of heat tolerant wheat variety in Uzbekistan (**a**) and Indian traditional wheat (**b**)

as for grain production. Recent releases such as Mackellar (Mackellar – virus beating wheat), Tennant, Brennan and Rudd are resistant to the most serious diseases in the wheat belt.

The goal of sustainable plant breeding is the long-term environmental and economic health of farming systems by producing wheat crops with safe and high nutritional value coupled with heat tolerance. Thus, an important goal for genetic improvement of agricultural crops is to adapt existing food crops to increasing temperatures, decreased water availability in some locations and flooding in others, rising salinity, and changing pathogen and insect threats. Such improvements will require diverse approaches that will enhance the sustainability of farms. It includes the research and breeding priorities for traditional and organic farming systems, farmer participatory breeding, converting wheat to a perennial crop, evolution of new heat tolerant wheat species; some progress has been made to create a heat-tolerant wheat variety in Uzbekistan (Fig. 1.6), conversion of C3 into C4 plants, low water and nitrogen-use efficiencies and non-GMO use of wild species for sustainable crop improvement are essential parameters in the pipe line of agricultural research.

Future sustainability can also be achieved by adopting strategies for bio-fortified staple plant foods to alleviate human dietary micronutrient needs and reduce global malnutrition. Poor people, especially infants and children, living at high food risk, need enriched foods with micronutrients added to major staple food crops such as rice, wheat, maize, beans and cassava. Micronutrient enrichment traits are available within the genomes of these major staple food crops but research is required for substantial increases in levels of Fe, Zn, vitamin A, carotenoids and other nutrients without negative effects on crop yield. The reduction of anti-nutrient substances which inhibit micronutrients increase in these food crops are to be integrated in breeding strategies. In this regard a significant success has been achieved by the creation of Golden Rice.

1.12 Conclusions and Prospects

To feed the world population which is increasing by 160 people per minute, with >90 % of them living in developing countries, will require an amazing increase in food production. Wheat may become the most important cereal in the world leaving behind rice and maize and these crops will account for >80 % in developing countries. Access to a range of genetic diversity is critical to the success of breeding programs to fight world hunger. The introgression of genes that created dwarf stature and increased disease/viral resistance in wheat provided the foundation for the Green Revolution (Borlaug 1997), demonstrated that genetic resources have enormous potential to increase production. Wheat hybrids and synthetics may provide the yield increases needed in the future. *Tripsacum*, a wild relative of maize, represents an untapped genetic resource for abiotic and biotic stress resistance. The application of molecular and genetic engineering technologies enhances the use and exploitation of genetic resources. The effective and complementary use of all molecular, technological tools and resources will be required for meeting the challenge posed by the world's expanding demand for food by 2050. Thus, important strategic goals are required for genetic improvement in existing agricultural food crops to mitigate against climate change i.e. increasing temperature, decreased water availability/flooding in other regions, increasing salinity, changing pathogen (disease, insect pests) threats and performance at low levels of fertilizer and other chemical inputs. Such improvements will require diverse approaches that will enhance the sustainability of farms. These goals include more effective land and water use policies, integrated pest management approaches, reduction in harmful inputs, and the development of a new generation of agricultural crops tolerant of diverse types of stress.

Plant breeders, particularly those at public institutions, have an interest in reducing negative impacts of agriculture and improving the natural environment to provide and maintain ecosystem services (e.g. clean soil, water, and air, carbon sequestration), and to create new agricultural paradigms. Plant breeding can be a powerful tool in the near future to bring harmony between agriculture and the environment, but partnerships between plant breeders, ecologists, urban planners, and policymakers are needed to make this a reality. Sustainable agriculture, reduces harm to the environment through the reduction/elimination of polluting substances such as pesticides and nitrogen fertilizers, water conservation practices, soil conservation practices, restoration of soil fertility, maintenance of agricultural biodiversity and biodiversity as a whole (Scialabba and Hattam 2002).

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Chapter 2
Breeding Crop Plants for Improved Human Nutrition Through Biofortification: Progress and Prospects

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Abstract Micronutrients are essential minerals and vitamins required by humans in tiny amounts which play a vital role in human health and development. Over three billion people in the world are malnourished, particularly in the developing countries. Current food systems cannot provide sufficiently balanced micronutrients required to meet daily needs and to sustain the wellbeing of people in developing countries. Heavy and monotonous consumption of cereal-based foods which contain limited amounts of micronutrients is one of the major reasons for the significantly high prevalence of micronutrient deficiencies in many of the developing countries. The development of crops with enhanced micronutrient concentration is one of the most sustainable and cost-effective approaches to alleviate micronutrient malnutrition globally. In this chapter we focus on the research to improve mineral element concentration in crops through plant breeding strategies, especially in major cereal crops and a legume which are most widely cultivated and preferred in Africa and Asia. Biofortification is an appropriate strategy to increase the bioavailable

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concentrations of an element in edible portions of crop plants through traditional breeding practices or modern biotechnology to overcome the problem of micronutrient deficiencies. Therefore, conventional breeding with modern genetic engineering approaches are important for developing crop cultivars with enhanced micronutrient concentrations to improve human health. This chapter reports on biofortification research on rice, pearl millet, sorghum, maize, wheat and common bean.

Keywords Biofortification • Bioavailability • Micronutrient deficiency • Micro nutrients • Fe • Zn

2.1 Introduction

For good health, humans require at least 49 essential nutrients to meet their metabolic needs (Table 2.1).

Insufficient ingestion of even one of these essential nutrients will result in adverse metabolic disturbances leading to sickness, poor health, impaired development in children and high economic costs to society (Branca and Ferrari 2002; Golden 1991; Grantham-McGregor and Ani 1999; Ramakrishna et al. 1999). Micronutrient deficiency is the lack of essential vitamins and minerals required in small amounts by the body for proper growth and development. Micronutrients are not limited to

Table 2.1 The 49 known essential nutrients for sustaining human life

Water and energy	Protein (amino acids)	Lipids-fat (fatty acids)	Macro elements	Micro elements	Vitamins
Water	Histidine	Linoleic acid	Na	Fe	A
Carbohydrates	Isoleucine	Linolenic acid	K	Zn	D
	Leucine		Ca	Cu	E
	Lysine		Mg	Mn	K
	Methionine		S	I	C
	Phenylalanine		P	F	B_1
	Threonine		Cl	B	B_2
	Tryptophan			Se	B_3
	Valine			Mo	Niacin
				Ni	B_6
				Cr	Folate
				V	Biotin
				Si	B_{12}
				As, Sn, Co (Cobalamin)	

Source: Welch and Graham (2002)

vitamins A, B, C and D, but also include, calcium, folate, iodine, iron and zinc. Common micronutrient deficiencies among children and lactating women include iron, iodine, vitamin D, selenium, vitamin A, folate and zinc. The Food and Agricultural Organization, United Nations, and the World Health Organization (FAO/WHO 2000) reported the daily required amounts for some of the essential nutrients for adults, which are listed in Table 2.2. Agricultural products are the primary source of all these nutrients. If agricultural systems fail to provide enough products containing adequate quantities of all nutrients during all seasons, the result is a dysfunctional food system that cannot support healthy lives. Unfortunately, this

Table 2.2 Recommended nutrient intakes for males and females between the ages of 25 and 50

Nutrient	Assessment	Male	Female
Energy (kcal)	AEA	2,900	2,200
Protein (g)	AEA	63	50
Vitamin A(μg retinol equivalent)	RDA	1,000	800
Vitamin D (μg)	RDA	5	5
Vitamin E (mg)	RDA	10	8
Vitamin K (μg)	RDA	80	65
Riboflavin (mg)	RDA	1.7	1.3
Niacin (mg)	RDA	19	15
Thiamin (mg)	RDA	1.5	1.1
Pantothenic acid (mgd^{-1})	ESADDI	4–7	4–7
Vitamin B_6 (mg)	RDA	2	1.6
Vitamin B_{12} (μg)	RDA	2	2
Biotin (μgd^{-1})	ESADDI	30–100	30–100
Folate (μg)	RDA	200	180
Vitamin C (mg)	RDA	90	60
Ca (mg)	RDA	800	800
P (mg)	RDA	800	800
Mg (mg)	RDA	350	280
Na (mg)	MR	500	500
K (mg)	MR	2,000	2,000
Cl (mg)	MR	750	750
Fe (mg)	RDA	10	15
Zn (mg)	RDA	15	12
Cu (mg)	ESADDIC	1.5–3.0	1.5–3.0
Se (μg)	RDA	70	55
I (μg)	RDA	150	150
Mn (μg)	ESADDI	2–5	2–5
Mo (μg)	ESADDI	75–250	75–250
Cr (μg)	ESADDI	50–200	50–200
F ((mg)	ESADDI	1.5–4.0	1.5–4.0

Source: FAO/WHO (2000)

AEA Average Energy Allowance, *RDA* Recommended Dietary Allowances, *ESADDI* Estimated Safe and Adequate Daily Dietary Intakes, *MR* Minimum Requirement

is the case for many agricultural systems in all developing countries (Graham et al. 2001; McGuire 1993; Schneeman 2001).

Micronutrient malnutrition has been designated as the most serious challenge to humanity (Bouis et al. 2011) because two-thirds of the world population is at risk of deficiency in one or more essential mineral elements (Stein 2010; White and Broadley 2009). The concern is more crucial in developing countries, especially among women, infants and children of resource-poor families. More than one-half of the total populations in developing countries are reported to be affected by micronutrient deficiency and therefore more susceptible to infections and impairment of physical and psycho-intellectual development (WHO 2005). The mineral elements most commonly lacking in human diets are iron (Fe) and zinc (Zn) (Stein 2010; White and Broadley 2009), whereas other essential minerals such as calcium (Ca), copper (Cu), magnesium (Mg), iodine (I) and vitamin A can be deficient in some human diets as well (Genc et al. 2005; White and Broadley 2005). These deficiencies are caused by customary diets that lack diversity (overly dependent on a single staple food), situations of food insecurity when populations do not have enough to eat (WHO 2002) as well as low intake of vegetables, fruits, and animal and fish products, which are rich sources of minerals. The widespread deficiencies of Fe and Zn in developing countries are mostly due to monotonous consumption of cereal-based foods with low concentrations and reduced bioavailability of Fe and Zn (Graham et al. 2001; Welch and Graham 1999). The recommended daily allowance (RDA) of both Fe and Zn is 12–15 mg for adults and 10 mg for children (FAO 2003; ICMR 2009). Both minerals have health and clinical significance as they affect growth and development and many physiological and neurophysiological functions (Sandstead 1994).

The causes of malnutrition among children and lactating women worldwide include:

(a) Inadequate maternal, prenatal and perinatal health care; poor prenatal diet,
(b) Premature infant birth; low or very low birth weight resulting in underdeveloped infants,
(c) Inadequate or no breastfeeding,
(d) Animal milk or milk products offered instead of fortified infant formula,
(e) Diluted or improperly prepared infant formula, which decreases the nutritional adequacy of the formula or introduces food safety risks,
(f) Premature introduction of solid foods to the infant diet,
(g) Insufficient amounts of food and/or lack of essential nutrient-rich foods,
(h) Insufficient feedings and/or inappropriate feeding practices in orphanages, particularly for children with special needs,
(i) Inadequate exposure to sunlight, which inhibits vitamin D production, a crucial vitamin that facilitates calcium absorption for bone growth,
(j) Cultural food practices introduced too early. For example, tea is often served with meals in many countries. Although tea has many health benefits, when consumed in large quantities as part of a nutrient-poor diet, naturally-occurring substances in tea may inhibit the absorption of important vitamins and minerals,
(k) Lack of fortified foods, beverages, and vitamin supplements due to high cost or unavailability,

(l) The stress of transitioning from birth mother to secondary care provider and then to the new family can disrupt a child's natural feeding cycle, resulting in nutritional issues (Adoption Nutrition- the go-to nutrition and feeding resource for adoptive and foster families www.adoptionnutrition.org/what-every-parent-needs-to know/contributing-factors-to-malnutrition).

Micronutrient malnutrition greatly increases mortality and morbidity rates, diminishes cognitive abilities of children and lowers their educational attainment, reduces labor productivity, stagnates national development efforts, contributes to continued high population growth rates and reduces the livelihood and quality of life for all those affected (Combs and Welch 1998; Welch and Graham 1999). In an attempt to reverse this scenario, research has been carried out to improve nutrient concentrations in edible crops by biofortification (Bouis et al. 2011; Mayer et al. 2008; Nestel et al. 2006; White and Broadley 2005). Biofortification can be achieved by combining breeding strategies with improved fertilization management (Bouis et al. 2011; Cakmak et al. 2010; Pfeiffer and McClafferty 2007; White and Broadley 2005). Biofortification of staple crops can be a sustainable and cost-effective approach to combat malnutrition (Bouis 1999; Meenakshi et al. 2010) especially of rural populations in remote, low-rainfall areas, with limited access to a diverse diet, commercially-fortified foods or supplements (Saltzman et al. 2013). Genetic variation of grain micronutrient densities in adapted genetic materials is the basic requirement for biofortification breeding programs, and thus needs to be assessed beforehand. Micronutrient-enriched crops can be obtained by conventional breeding or by biotechnological approaches (Brinch-Pedersen et al. 2007; Mayer et al. 2008). An understanding of the genetic basis of the accumulation of micronutrients in food grains and mapping of the quantitative trait loci (QTL) will provide the basis for devising plant-breeding strategies and to improve grain micronutrient content through marker-assisted selection (MAS). Developing micronutrient-enriched staple plant foods, either through traditional plant breeding methods or via molecular biological techniques, is a powerful intervention tool that targets the most vulnerable people (Bouis 2000; Combs Jr et al. 1996).

Studying the importance of malnutrition in developing and underdeveloped countries and also the availability of fortified crops in such countries is a major challenge for policymakers and researchers to provide the hungry world with nutrient rich foods. In many of the countries, agriculture is the main occupation and supplies food to the nation. Hence, biofortification of agriculturally-important crops like maize, rice, wheat, sorghum, pearl millet, manioc and common bean plays a major role in providing the essential micronutrients to this micronutrient deficient world.

This chapter mainly focuses on the genetic enhancement of crop plants for micronutrients with major focus on grain Fe and Zn in solving the problem of micronutrient deficiency through breeding major cereal crops like rice, wheat, pearl millet, sorghum, maize and common bean for improvement in grain yield associated with increased micronutrients. We discuss mainly the introduction and importance of micronutrients in human health. The consequences of deficiencies of micronutrients on human health with respect to Fe, Zn, iodine vitamin D, vitamin A, vitamin B, folate and selenium. We also discuss the genetic enhancement of crop plants for

micronutrients, mainly in rice, sorghum, pearl millet, maize and common bean, for the current status of genetic variability for various micronutrients content along with their association with yield and yield components. Later we also discuss the genetic and environmental effect on grain micronutrient content and also on marker-assisted selection and transgenic approaches used for biofortification. The chapter concludes with a statement on biofortification as an improved tool for human health.

2.2 Consequences of Micronutrient Deficiencies on Human Health

The importance of some micronutrients and their consequences on human health are discussed under the following headings.

2.2.1 Iron (Fe)

Iron is a micronutrient that is essential to the structure of every cell in the body, but particularly to red blood cells (hemoglobin), which transport oxygen in the blood to body tissues. In addition, iron is also a key component in proteins, in muscle tissue and is critical for the normal development of the central nervous system. Iron deficiency is the most common form of malnutrition worldwide. A lack of iron in the diet results in iron deficiency. The most commonly recognized condition associated with iron deficiency is anemia. Iron deficiency is a worldwide problem that is directly correlated with poverty and food insecurity. Approximately one-third of the world's population suffers from iron deficiency-induced anemia, 80 % of which are in developing countries (Boccio and Iyengar 2003) (Fig. 2.1). In iron deficiency, the amount of iron stored for later use is reduced as indicated by a low serum ferritin level, but has no effect on the iron needed to meet the daily needs of an individual. If the body requires increased iron (due to a rapid growth spurt, for example), a person with inadequately stored iron has no reserves to use. When the body lacks sufficient iron to make adequate hemoglobin, red blood cells cannot transport sufficient oxygen to tissues throughout the body. This can cause iron-deficiency anemia, an advanced stage of iron deficiency. Iron is also critical for normal cardiac and skeletal muscle function and is a key component of enzymes involved in brain development. The major causes of iron deficiency are inadequate iron intake/availability from foods and blood loss or increased demand due to disease (e.g. malaria, HIV/AIDS) (Lemke 2005; Rosegrant and Cline 2003; Skalicky et al. 2006).

The consequences of iron deficiency include increased mortality and morbidity rates, diminished cognitive abilities of children, and reduced labor productivity that in turn stagnate national development (Caballero 2002). Fe deficiency in pregnant women may cause irreversible damage to fetal brain development leading to irreversible damage to intellectual development in their children (Gordon 1997). The developed world

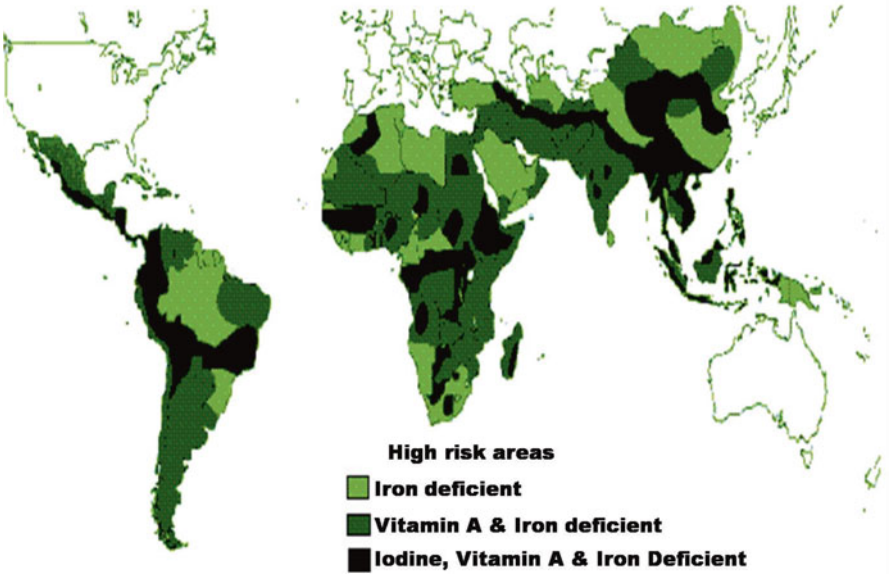

Fig. 2.1 World map indicating the world population is affected from iron deficiency (Source: Sanghvi (1996))

has made tremendous progress in alleviating micronutrient deficiencies through dietary diversification, processed food fortification, improved public health care and supplementation. In developing countries, these strategies are often too expensive and difficult to sustain. Treatment for iron deficiency includes oral iron supplementation that can be used for both prevention and treatment of iron deficiency anemia. Oral iron supplements are usually best absorbed on an empty stomach. However, because iron can irritate a child's stomach, supplements may need to be taken with food. A source of vitamin C, like citrus juice, enhances iron absorption. It usually takes several months of iron supplementation to correct the deficiency; iron also is rich in foods such as meats, poultry and fish, fortified cereals and oatmeal, legumes (e.g. soybeans and lentils), leafy greens and seeds (e.g. sesame and pumpkin).

2.2.2 Zinc (Zn)

Zinc is an essential mineral found in over 200 enzymes that are involved in a wide range of body functions. These zinc-containing enzymes play a role in immune function, wound healing, and making DNA and other proteins. Zinc supports normal growth and development during childhood and adolescence, and is required for a proper sense of taste and smell. Because zinc plays so many roles in the body, including brain development, a deficiency of zinc can impact multiple bodily functions and result in a wide variety of symptoms. Zinc deficiency alone is a major cause of child

death in the world, and responsible for nearly 450,000 children deaths (4.4 % of the children deaths per year globally) under 5 years of age (Black et al. 2008). Deficiency of zinc in the human body will result in a number of cellular disturbances and impairments such as immune dysfunctions and high susceptibility to infectious diseases, retardation of mental development, altered reproductive biology, gastrointestinal problems and stunted growth of children, reduced growth and, sexual maturity and weakened immune defense system (Black et al. 2008). Zinc deficiency can also contribute to vitamin A deficiency, since lack of zinc impairs the synthesis of the retinol-binding protein. Low dietary zinc intake (in general) is the main cause of zinc deficiency. The risk of zinc deficiency is particularly high in populations which depend on diets with low levels of absorbable zinc and with no or only limited access to sources rich in bioavailable zinc such as meat. Zinc deficiency is a problem particularly in regions where the population consumes mainly cereals and where soils are low in phytoavailable zinc (Cakmak 2008). Kim et al. (1998) showed that marginal zinc deficiency lowers the lymphatic absorption of vitamin E (α-tocopherol) in rats. Thus, intestinal absorption of vitamin E is reduced by low-zinc status. Zinc deficiency can be managed by supplements (zinc sulfate or zinc gluconate), increasing dietary intake, vitamin and mineral supplements to aid in zinc absorption (e.g. A, E, B6, magnesium, phosphorous and calcium). Foods high in zinc include meats and seafood, eggs, whole grains and oats, nuts and seeds, leafy greens, vegetables, herbs and yogurt.

2.2.3 Iodine (I)

Iodine is a nutrient essential for normal functioning of the thyroid gland, production of thyroid hormones and metabolism. Iodine deficiency is the world's most common, but preventable, deficiency and a cause of mental retardation. Iodine deficiency is common in areas where there is little iodine in the diet particularly in remote inland areas where no marine foods are eaten and in mountainous regions of the world where food is grown in iodine-poor soil. Iodine is typically found in small amounts in food and varies depending on environmental factors such as the soil concentration of iodine and the use of fertilizers. Prevention includes adding small amounts of iodine to table salt, a product known as iodized salt. Iodine compounds have also been added to other foodstuffs, such as bread (fortified), dairy products (e.g. cheese, cow milk and yogurt), soy milk, soy sauce and seafood. A meta-analysis found that iodine supplementation improves some maternal thyroid indices and may benefit aspects of cognitive function in school-age children, even in marginally iodine-deficient areas (Taylor et al. 2014). Iodine is not produced by the body, so it must be obtained through diet. Sufficient thyroid hormone is not produced without enough iodine. Iodine deficiency can lead to enlargement of the thyroid (goiter), hypothyroidism, and mental retardation in infants and children whose mothers were iodine deficient during pregnancy. Iodine deficiency resulting in goiter occurs in 187 million people globally as of 2010 (2.7 % of the population) (Vos et al. 2012). It resulted in 2,700 deaths in 2013 up from 2,100 deaths in 1990 (GBD 2013). Consuming foods high in iodine can help treat and prevent iodine deficiency

(http://www.orphannutrition.org/understanding-malnutrition/micronutrient-malnutrition/#iodin).

2.2.4 Vitamin D

Vitamin D is a fat-soluble vitamin naturally produced in the body. It is essential to the absorption of calcium for proper bone development and function. Vitamin D is found in cod and cod liver oil, egg yolks, milk and butter, fortified cereals and salmon and shrimp. Hypovitaminosis D is a deficiency of vitamin D, which can lead to abnormalities in bone development and a condition in children called rickets, wherein, bones become soft and may bend, distort and/or fracture. It is one of the most common childhood diseases in many developing countries. Treatment of rickets involves vitamin D supplementation, increasing dietary intake of calcium, phosphates, and vitamin D, daily exposure to small amounts of sunlight (15 min/day for lighter-skinned children; longer for darker-skinned children), special braces to position the bones (severe cases), surgery (very severe skeletal deformities) (http://www.orphannutrition.org/understanding-malnutrition/identifying-malnutrition-in-orphans/#vitamin D).

Emerging evidence suggests that vitamin D plays a role in non-alcoholic fatty liver disease (NAFLD) pathogenesis (Eliades et al. 2013). NAFLD is one cause of a fatty liver, occurring when fat is deposited (steatosis) in the liver due to causes other than excessive alcohol use. NAFLD is the most common liver disorder in Western industrialized nations (Shaker et al. 2014).

2.2.5 Vitamin A

Vitamin A is a group of compounds that play a significant role in vision, bone development, immune support and normal bodily function. Retinol and beta-carotene are forms of pre-vitamin A which are converted to vitamin A in the body. Deficiency is a common problem in developing countries, but rarely seen in developed countries. In Africa, vitamin A deficiency (VAD) affects more than 30 million children, is a contributing factor to 10.8 million deaths overall and causes blindness in another 2.55 million annually. VAD is estimated to affect approximately one-third of children under the age of 5 around the world. It is estimated to claim the lives of 670,000 children under the age of 5 annually (WHO 1995–2005). Approximately 250,000–500,000 children in developing countries become blind each year owing to VAD, with the highest prevalence in Southeast Asia and Africa (Black et al. 2008). According to the World Health Organization, VAD is under control in the United States, but in developing countries is a significant concern. Nyctalopia (night blindness) is one of the first signs of VAD, later it can lead to xerophthalmia, keratomalacia and complete blindness since Vitamin A has a major role in phototransduction. As elucidated by Sommer et al. (1986), vitamin A

deficiency leads to increased risk in children of developing respiratory and diarrheal infections, decreased growth rate, slow bone development and decreased likelihood of survival from serious illness. Treatment for vitamin A deficiency includes oral and injectable supplementation, food fortification and increasing consumption of vitamin A-rich foods from animals, fruits and vegetables.

2.2.6 Vitamin B12

Vitamin B12 is a water-soluble vitamin that exists in several forms. Vitamin B12 is needed for proper red blood cell formation and the maintenance of healthy nerve cells. It is also essential in making DNA, the genetic material in cells. Vitamin B12 is found in fortified cereals and occurs naturally in foods coming from animals, including fish, meat poultry, eggs, milk and milk products. Vitamin B12 deficiency, also known as hypocobalaminemia, refers to low blood levels of vitamin B12 (Herrmann 2011). Deficiency leads to a wide variety of signs and symptoms including a decreased ability to think and changes in personality such as depression, irritability, psychosis, abnormal sensations, changes in reflexes, poor muscle function, inflammation of the tongue, decreased taste, low red blood cells, reduced heart function and decreased fertility (Hunt et al. 2014). Without early treatment some of the changes may be permanent (Lachner et al. 2012). Increased requirements occur in HIV/AIDS and in those with rapid red blood cell breakdown (Hunt et al. 2014). Diagnosis is typically based on vitamin B12 blood levels below 120–180 picomol/L (normal level, 170–250 pg/mL) in adults. Once identified it is easily treated with supplementation by mouth or injection (Vidal et al. 2005), nasal sprays and increased consumption of animal products. Plants which provide vitamin B12 include vegetables and fortified cereal foods with meat, fish and eggs.

2.2.7 Folate ($C_{19}H_{19}N_7O_6$)

Folate, also known as vitamin B9, is a water-soluble vitamin naturally occurring in foods. Folate is necessary for the production and maintenance of new cells and is especially important during periods of rapid cell division and growth, such as infancy and pregnancy. Both adults and children need folate to make normal red blood cells and prevent anemia. Folate is involved in adenosine, guanine and thymidine synthesis (part of DNA synthesis). Insufficient quantities cause the medicinal condition of folate deficiency anemia (Huether et al. 2004). Initial symptoms of deficiency are loss of appetite and weight; additional signs are weakness, sore tongue, headache, heart palpitation, irritability and behavioral disorders. In adults, anemia (macrocytic, megaloblastic anemia) can be a sign of advanced folate deficiency (Haslam and Probert 1998). Folate occurs naturally in leafy greens (e.g. spinach and turnip greens), peas, beans, fruits and other vegetables. Folic acid (synthetic folate) is commonly added to

enrich grain products such as cereals, rice, pasta, bread and flour. Inadequate dietary intake of folate can slow growth rate in infants and children. Folic acid is available in most multivitamins and in some foods. Supplementing the diet with vitamins and foods rich in folate or folic acid can help prevent and treat folate deficiency.

2.2.8 Selenium (Se)

Selenium is a trace mineral needed in small amounts by the human body for good health. It is incorporated into proteins to make important antioxidant enzymes. These enzymes help prevent cellular damage from free radicals that can cause the development of chronic diseases such as cancer and heart disease. Selenium can be found in foods such as Brazil nuts, tuna, cod fish, beef, poultry, enriched pasta, rice, eggs, cottage cheese and oatmeal. In the USA, the Dietary Reference Intake for adults is 55 μg/day. In the UK it is 75 μg/day for adult males and 60 μg/day for adult females. The 55 μg/day recommendation is based on full expression of plasma glutathione peroxidase. Selenoprotein P (Papp et al. 2007) is a better indicator of selenium nutritional status and full expression of it would require more than 66 μg/day (Xia et al. 2005). Selenium deficiency is a result of inadequate selenium in the diet. Though rare, it can lead to three specific diseases: Keshan disease results in an enlarged heart and poor heart function in selenium-deficient children. Kashin-Beck disease results in osteoarthritis and weakened immune system in children (Moreno et al. 1998). Myxedematous endemic cretinism results in mental retardation in infants born to mothers deficient in both selenium and iodine. Selenium supplementation protects people from developing Keshan disease but cannot reverse heart muscle damage once it occurs. There is little evidence that improving selenium nutritional status prevents Kashin-Beck disease. It can occur in patients with severely compromised intestinal function, those undergoing total parenteral nutrition, those who have had gastrointestinal bypass surgery and also in individuals of advanced aged (e.g. over 90) (Ravaglia et al. 2000). Selenium is also necessary for the conversion of the thyroid hormone thyroxine (T4) into its more active counterpart, triiodothyronine and as such a deficiency can cause symptoms of hypothyroidism, including extreme fatigue, mental slowing, goiter, cretinism and recurrent miscarriage (http://www.atsdr.cdc.gov/toxprofiles/tp92-c3.pdf).

2.3 Genetic Enhancement of Crop Plants for Micronutrients

The success of any crop improvement program depends on the magnitude of genetic variability and the extent to which the desirable trait is heritable. The estimate of variability of yield and yield-contributing characters and their heritable components in the material is important in any crop breeding program. The presence of genetic variability in breeding material has been emphasized by Falconer

(1981), so as to exercise critical selection pressure. Information on the nature and magnitude of variation in the segregating population of a cross where selection is actually practiced will be more meaningful and is of immediate practical utility. Moreover, correlation studies provide information about the relative contribution of various component traits on grain yield per plant and help in effective identification and selection of superior plants. Since yield is polygenically controlled and highly influenced by environment, selection based on yield alone is not effective. Therefore, improvement in yield can be brought about by effecting indirect selection through yield attributes whose heritability is high and shows strong association with yield.

Genetic variability studies provide information about the extent of variation present in a population. The phenotypic variance measures the magnitude of variation arising out of difference in phenotypic values, while the genotypic variance measures the magnitude of variation due to differences in genotypic values. The absolute values of phenotypic and genotypic variances cannot be used for comparing the magnitude of variability for different characters since the mean and units of measurement of the characters may be different. Hence, the coefficients of variation expressed at the phenotypic and genotypic levels have been used to compare the variability observed among different characters. Although the genotypic coefficient of variation indicates the amount of genetic variability present in the character, the heritability estimates aid in determining the relative amount of heritable portion of variation. However, heritability values themselves provide no indication of the amount of genetic progress that would result from selecting the best individuals.

In recent years, the cognizance of genetic diversity and the evolutionary history of crop plants have yielded major advances in crop improvement. The measure of genetic divergence reveals the differences in gene frequencies. Mahalanobis's generalized distance estimated by the D^2 statistic (Rao 1952) is a unique tool for discriminating populations by considering a set of parameters together. In addition to estimation of variability, cognizance of the genetic diversity of the germplasm is necessary for effective choice of parents in hybridization. Knowledge of the amount of genetic variability present in a crop species with respect to yield and its attributes and their association, which reflects the nature and degree of relationship between any two measurable characters, is of great importance in achieving genetic improvement in that crop.

Biofortification breeding of crop plants focuses on improving grain Fe and Zn content. In a few studies researchers also have given importance to other micronutrients such as iodine and selenium. Genetic variability for micronutrient content in crop plants varies widely and micronutrient accumulation in grain also depends on agronomic practices, soil nutrient composition, environmental features and the variety or hybrid of each particular crop. In the following crops we discuss the genetic variability for grain Fe and Zn content, heritability, genes controlling the traits and so on, in individual crops with suggested breeding methods for biofortification programs.

2.3.1 *Rice (*Oryza sativa*)*

Rice is central to the lives of billions of people around the world. Possibly the oldest domesticated grain (~10,000 years), it is the staple food for 2.5 billion people (Anon 2004) and growing rice is the largest single use of land for food production, covering 9 % of the earth's arable land. Rice provides 21 % of global human per capita energy and 15 % of per capita protein (Anon 2002). Calories from rice are particularly important in Asia, especially among the poor, where it accounts for 50–80 % of daily caloric intake. As expected, Asia accounts for over 90 % of the world's production of rice, with China, India and Indonesia producing the most. Around 85 % of the rice that is produced in the world is used for direct human consumption (Anon 2002). Rice can also be found in cereals, snack foods, beverages, flour, oil, syrup and religious ceremonies to name a few other uses.

Rice belongs to the genus *Oryza* and has 2 cultivated and 22 wild species; the cultivated species are *O. sativa* and *O. glaberrima. Oryza sativa* is grown all over the world while *O. glaberrima* has been cultivated in West Africa for the past ~3,500 years (Anon 2002). Rice is grown under many different conditions and production systems worldwide, but most commonly in flooded fields. It is the only cereal crop that can grow for long periods of time in standing water (Anon 2004).

Rice is the world's most important food crop and a primary source of food for more than one-half the world's population. It is the predominant staple food crop for 15 countries in Asia and the Pacific, 10 in Latin America and the Caribbean, 7 in sub-Saharan Africa and 1 country in North Africa (FAO 1999). In developing countries, rice accounts for 715 kcal per capita per day, 27 % of dietary energy supply, 20 % of dietary protein and 3 % of dietary fat. Southeast Asian countries are heavily reliant upon rice. India accounts for nearly one-fourth (22 %) of the world's rice production, with China the leader. World rice production currently is around 597.8 million mt grown over 151 million ha with a productivity of 3.96 mt ha^{-1}. India has an area of 44 million ha under rice cultivation with an output of 99 million mt, which averages to a yield of around 2.10 mt ha^{-1}. Dietary intake surveys from China and India reveal an average adult intake of about 300 g of raw rice per day (FAO 1998). Technological advances during the last 40 years have led to an increase in rice production by 150 %. Rice production needs to increase even further to meet growing demand. Sustainable production will have to overcome a number of challenges including the decline in arable land, global water shortage and global climate change (Royal Society 2009).

Wide genetic variation exists for grain Fe and Zn content in rice germplasm accessions and this variation can be exploited in breeding programs to enhance Zn content in the grains (Graham et al. 1999; Welch and Graham 2004). A recent study by Gangashetty et al. (2013) screened germplasm accessions from the Western Ghats of Karnataka of non-basmati aromatic genotypes of rice for Fe and Zn content and found a range from 2–17.49 to 9.80–32.44 ppm, respectively. Anarudha et al. (2012) screened rice germplasm for Fe and Zn content and found Fe concentration ranged from 6.2 to 71.6 ppm and Zn from 26.2 to 67.3 ppm. Neelamraju

et al. (2012) reported the Fe concentration in brown rice ranged from 6 ppm in Athira to 72 ppm in *Oryza nivara* and Zn concentration from 27 ppm in Jyothi to 67 ppm in *O. rufipogon*. Significant genetic variation was reported for Fe and Zn in *indica* and aromatic rice varieties (Brar et al. 2011). Another study showed wide variation for micronutrient levels recorded among 46 tested rice genotypes, which ranged from 4.82 to 22.69 µg/g for grain Fe and 13.95–41.73 µg/g for grain Zn content (Banerjee et al. 2010). Liu et al. (1995) reported Zn content in grains of rice ranged from 0.79 to 5.89 mg/100 g with an average of 3.34 mg/100 g in a study done among 57 rice varieties. Qui et al. (1995) reported a higher variability in mineral contents in some rice cultivars and the level of Fe content varied from 15.41 to 162.37 mg kg^{-1} and Zn from 23.92 to 145.78 mg kg^{-1}.

*2.3.2 Pearl Millet [*Pennisetum glaucum *(L.) R. Br.]*

Pearl millet is the staple cereal of what is undoubtedly the harshest of the world's major farming areas: the arid and semiarid regions stretching over 7,000 km from Senegal to Somalia. There, on the hot, dry, infertile sandy soils having low organic matter content, farmers produce some 50 % of the world's pearl millet grain. The agricultural research challenge is how to help farmers in this often drought-devastated zone, living on the edge of the world's largest desert, who have no access to irrigation, affordable mineral fertilizer, pesticides or other purchased inputs. The answer may lie in their age-old staple, pearl millet. Indeed, there is probably no better cereal to relieve the underlying threat of starvation in the Sahelian and northern Sudanian areas extending from Mauritania, Senegal and The Gambia in the west, to eastern and northeastern Kenya and the coastal lowlands of Yemen, Oman, and Iran. Millions of people entrust their daily lives to this single species and of all the inhabitants on the planet, they are among the poorest in economic terms and most in need of help. Yet, at the moment, pearl millet continues to suffer from neglect and misunderstanding, in part because the crop grows in some of the poorest countries and regions, and in some of the least hospitable habitats for humans and livestock. People have therefore unjustly stigmatized it as a poor crop, fit only for temporary support of poor people until something better is identified.

Pearl millet is the sixth most important of the world's cereals. Descended from a wild West African grass (also *Pennisetum glaucum*), it was domesticated more than 4,000 years ago, probably in what is now the heart of the Sahara Desert. In ancient times, it was dispersed from its homeland to East Africa and thence to India, reaching there more than 3,000 years ago. Both regions adopted it eagerly and it has become a much-favored staple food grain, feed and fodder crop. Today, pearl millet is sown on ~22 million ha in Africa and ~12 million ha in Asia, as well as more than 3 million ha in Latin America, much of it in Brazil where it serves as the best available mulch component of sustainable limited-tillage soybean production on acid soils in the Cerrado region. Global production of pearl millet grain probably exceeds 20 million mt annually, to which India contributes nearly one-half. At least 200

million people depend on pearl millet for at least several months each year and a large percentage of them depend upon it throughout the year.

Pearl millet's important characteristic is its concomitant ability to withstand heat, low soil fertility and low moisture availability (Gupta et al. 2015). Today, approximately 40 % of the world's pearl millet is grown in Africa and the rest mostly contributed by India. About 85 % of Africa's production is in the West African countries, including Nigeria (5 M ha), Niger (7 M ha), Burkina Faso (1.5 M ha), Chad (3 M ha), Mali (1.5 M ha) and Senegal (1 M ha). Sudan (2 M ha), Tanzania (0.2 M ha), Eritrea, Namibia and Uganda (0.1 M ha each) are other producing countries in Africa. In these regions, pearl millet is a staple food of more than 90 million people. Pearl millet is a highly nutritious cereal with high levels of metabolizable energy and protein, and a more balanced amino acid profile (Andrews and Kumar 1992). Pearl millet grains from crops grown with 20–40 kg ha^{-1} of applied nitrogen have 10–11 % protein, comparable to the protein found in wheat cultivars. Processing technologies for preparing various types of alternative and health food products have been developed. These products have been shown to have lower glycemic index levels than similar products produced from wheat (Sehgal et al. 2004), thus increasing the food value of pearl millet for those prone to diabetes. Pearl millet grains lack gluten, unlike most of the major cereals, thus enhancing its health value for those allergic to gluten (Dahlberg et al. 2004).

Pearl millet is less prone to aflatoxin contamination than sorghum and maize. Collins et al. (1997) reported that eggs produced by chickens fed pearl millet-based diets have lower levels of low-density lipoprotein, thus making possible the production of *designer* eggs for those with high cholesterol. These findings suggest that pearl millet can play an important role not only in contributing to the nutritional security of the poor in the pearl millet growing areas of India and Sub-Saharan Africa, but could also have potential health value for the affluent.

Pearl millet has both natural relatively high concentrations of Fe and Zn with demonstrated potential to increase these levels further with plant breeding. Several reports indicate the existence of large variability for grain Fe and Zn in various types of genetic materials of pearl millet. For example, a recent study showed for all tested minerals a moderate to high range in mineral density among the West and Central Africa (WCA) pearl millet accessions studied (Burger et al. 2014). The study focused on the grain density of several minerals in 225 Sudanese pearl millet accessions evaluated in Sudan also found wider density ranges for all 8 minerals (Bashir et al. 2014). A study conducted with a limited number of 27 genotypes at ICRISAT showed high levels and large variability of both Fe (40–580 ppm) and Zn (10–66 ppm) in pearl millet grains (Jambunathan and Subramanian 1988). Other studies on grain Zn and Fe densities in pearl millet material, based on means of two environments reported from India, ranged around 30–80 mg kg^{-1} Fe and 20–70 mg kg^{-1} Zn (Govindaraj et al. 2013; Velu et al. 2007). Parthasarathy Rao et al. (2006) reported that in the major pearl millet growing states of India, pearl millet accounts for the largest share of Fe and Zn intake by the population, and it is also the cheapest source of these micronutrients as compared to other cereals and even vegetables. Pearl millet is a significant source of these micronutrients both in India and Sub-Saharan Africa.

2.3.3 *Maize (*Zea mays *L.)*

Maize is a major component of the daily diet of many of the neediest people of the world, and was selected as a target crop by the HarvestPlus Biofortification Program (Nestel et al. 2006). Maize is a major cereal crop widely consumed in developing countries, which have a high incidence of iron deficiency anemia. The major cause of Fe deficiency in these countries is inadequate intake of bioavailable Fe, where poverty is a major factor. Therefore, biofortification of maize by increasing Fe concentration and/or bioavailability has great potential to alleviate this deficiency. Maize is also a model system for genomic research and thus allows the opportunity for gene discovery. The development of an efficient breeding program to increase mineral concentrations in maize depends on the presence of genetic variability in this species. A study evaluating the kernel Fe and Zn of 67 diverse maize genotypes grown during 2006–2008 indicated significant variation for both micronutrients. Kernel Fe concentration in 2006 varied from 20.38 to 43.79 mg/kg, whereas the same ranged from 23.23–54.29 to 29.22–49.24 mg/kg, in 2007 and 2008, respectively. Kernel Zn varied from 15.06–29.88, 7.01–22.01 to 13.64–26.54 mg/kg, in 2006, 2007 and 2008, respectively (Agrawal et al. 2012). Queiroz et al. (2011) reported significant variability in the contents of Zn (17.5–42 $mgkg^{-1}$) and Fe (12.2–36.7 $mgkg^{-1}$) in 22 tropical maize inbred lines with different genetic backgrounds. Significant differences in the Fe and Zn concentrations in maize have been reported in many genotypes in trials conducted in Mexico and Zimbabwe by Banziger and Long (2000) and in Nigeria by Menkir (2008). Fe and Zn concentrations of more than 1,000 CIMMYT improved maize genotypes and 400 *core accessions* (landraces) from different environments were analyzed and little variation of Fe levels in grain (average 2,075 mg/g) and moderate variation for Zn concentration in grain (mostly 15–35 mg/g) were reported (Banziger and Long 2000; Long et al. 2004). Hence maize also serves as a major food source for Fe and Zn in many parts of the world.

2.3.4 *Sorghum [*Sorghum bicolor *(L.) Moench]*

Sorghum is an affordable staple food for more than 400 million people in Africa and some parts of Asia, many of whom live in the drier, more vulnerable agricultural areas. However, sorghum is deficient in most essential nutrients, and it is difficult to digest when cooked. If enhanced with key nutrients it could benefit key targeted populations who suffer from micronutrient deficiency. Sorghum is a crop with many advantages; it grows quickly and can tolerate much more heat and drought than most other crops. Sorghum also is gluten free and can be a good substitute for wheat in baked goods and other products. In Africa, sorghum is used to make bread and nutritious porridge, and can even be popped like corn. Sorghum is an important crop in Africa, with 23.4 million mt produced in 2012. While world production of sorghum appears to be level, production is slowly increasing in Africa.

In an attempt to create a sorghum database for grain Fe and Zn content at ICRISAT, Ashok Kumar et al. (2012) evaluated the ICRISAT germplasm core collection, improved varieties and partner institution selected varieties. In this study the range for Fe was 8–192 mg kg^{-1} and 14–91 mg kg^{-1} for Zn in the landraces. In a more recent study Ashok Kumar et al. (2013) at ICRISAT-India studied three particularly-derived diallel crosses for combining grain Fe and Zn content and also studied the heterosis for grain Fe and Zn content. Results indicated a large exploitable genetic variability available in sorghum germplasm and also observed the heterosis for grain Fe and Zn content without affecting the yield. This study indicated that the expression of grain Zn concentrations in sorghum is governed predominantly by additive gene effects, suggesting the high effectiveness of progeny selection in pedigree selection or population breeding to develop lines with increased levels of grain Zn concentrations, while the grain Fe concentration is governed predominantly by non-additive gene effects in combination with additive gene effects, suggesting scope for heterosis breeding in addition to progeny selection to develop lines with increased levels of grain Fe concentrations. The performance of the crosses can be predicted based on general combining ability (GCA) for grain Zn but information on both GCA and specific combining ability (SCA) is required for Fe. There is scope for exploitation of heterosis to improve grain Fe content. Some of the crosses developed in the study significantly outperformed parents for Fe and Zn concentration with no yield loss, indicating that it is possible to develop high grain Fe and Zn cultivars in high-yielding backgrounds. Nguni et al. (2011) evaluated sorghum genotypes of improved and farmers varieties from southern Africa for grain Fe and Zn; analysis ranged from 2.74–8.18 mg/100 g to 2.03–5.53 mg/100, respectively. The availability of wide genetic variability for grain Fe and Zn content in sorghum will help breeders select superior genotypes with high yield while improving micronutrients content.

2.3.5 *Phaseolus Bean (*Phaseolus vulgaris *L.)*

The common bean is the most important economic variety of the genus *Phaseolus* and is grown throughout the world. It requires much warmth and sun; cool weather and wind hamper growth. The crops prefers moderately-heavy or light soils are preferred. It is the most important legume worldwide for direct human consumption. The crop is consumed principally for its dry (mature) beans, shell beans (seeds at physiological maturity) and green pods. When consumed as seed, beans constitute an important source of dietary protein (22 % of seed weight) that complements cereals for over one-half billion people, mainly in Latin America. The largest producers of dry beans are Brazil, Mexico, China and the USA. Annual production of green beans is around 4.5 million mt, with the largest production taking place around the Mediterranean and in the USA. The common bean was used to derive important principles in genetics.

The degree of genetic variability present in Fe and Zn concentrations in common beans seeds was observed by researchers at the International Center for Tropical Agriculture (CIAT). A core collection of over 1,000 accessions of common beans were evaluated (Beebe et al. 2000), and showed a range in Fe concentrations from 34 to 89 $\mu g g^{-1}$ Fe (average 55 $\mu g g^{-1}$ Fe) while the Zinc concentrations in these same accessions ranged from 21 to 54 $\mu g g^{-1}$ Zn (average 35 $\mu g g^{-1}$Zn) (Graham et al. 1999). Recently, some common bean accessions from Peru were found to contain high levels of Fe averaging over 100 $\mu g g^{-1}$Fe. The results showed that there is sufficient genetic variability available to increase significantly Fe (~80 %) and Zn (~50 %) concentrations in common beans.

2.3.6 *Breeding Strategies*

A common breeding strategy can be used to enhance micronutrient content in crop plants based on their pollination systems (Fig. 2.2). Applied breeding programs begin with introduction of material developed elsewhere for improved micronutrient content. Advanced breeding lines, released varieties and hybrids also can be used as base material for developing new elite lines with trait breeding for micronutrients. Availability of genetic variability in the population can be used at the beginning to harness the genetic variability for developing new breeding lines. If the available genetic variability is not sufficient to develop the breeding lines, then it can be created by hybridization, mutation and polyploidy breeding approaches. A

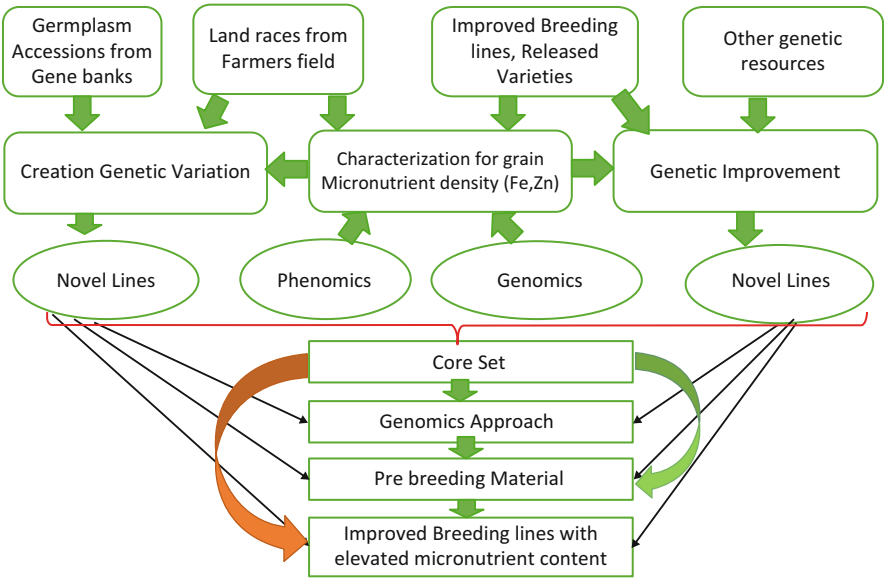

Fig. 2.2 Breeding strategy for micronutrient enhancement in crop plants

core set of genetic germplasm will be developed by evaluating complete genetic material and breeding lines available in a particular crop. At present molecular marker-assisted breeding is gaining importance in fast-track breeding for developing genetic material. The combination of molecular breeding and conventional breeding will be of great help in developing the genetic material and elite breeding lines in the shortest time available. Based on the pollination systems in crop plants, breeding methods can be applied. Breeding methods used in rice, sorghum and beans include mass selection, pedigree selection, single seed descent method of selection, backcross breeding, mutation breeding and marker-assisted selection. The breeding methods commonly followed in maize and pearl millet include population improvement approaches, mass selection and marker-assisted selection. If a crop is often cross-pollinated, like sorghum, either of the selection methods used for self-pollinated and cross-pollinated selection methods can be practiced depending upon the breeding objectives.

2.4 Effect of Genetics and Environment on Grain Micronutrient Content

Genotype by environment (G × E) interaction is the differential response of crop genotypes to changing environmental conditions. Such interactions complicate testing and selection in breeding programs and result in reduced overall genetic gains of desired traits (Shafii and Price 1998). Understanding the G × E interaction therefore allows the making of informed choices regarding which locations and input systems to use in the breeding efforts. Burger et al. (2014) reported significant G × E interaction effects for grain Fe and Zn densities in WCA pearl millet, showing the importance of multi-environmental evaluation to identify genotypes stable across environments. Studies on pearl millet in general have shown a significant G × E interaction effect for grain Fe and Zn densities as well (Govindaraj et al. 2013; Gupta et al. 2009; Velu et al. 2011), indicating the general importance of basing biofortification breeding programs on multiple environment testing.

Environment, genotype and G × E interaction significantly affected Fe concentration in rice grains (Anuradha et al. 2012; Suwarto and Nasrullah 2011). The pH, organic matter content and Fe/Zn levels of native soils showed significant effects on grain Fe and Zn content in rice (Chandel et al. 2010). Comparative analysis of grain nutrient contents (Fe and Zn) of genotypes grown in three locations showed significant differences, thus indicating a strong influence of native soil properties on Fe and Zn levels in grain (Banerjee et al. 2010). Several studies carried out in The Philippines, Bangladesh, Korea and Vietnam have reported a significant G × E interaction effect on grain nutritive-value related traits in rice, including factors, such as, wet and dry season, inherent soil properties like saline, acidic or neutral soils, nitrogen supply and period of flooding during crop growth (Gregorio et al. 2000).

In wheat, significant G × E interactions on grain nutrients were reported, demonstrating the importance of environmental effects on Fe and Zn concentrations (Badakhshan et al. 2013). Several studies reported significant G × E interactions for grain nutrient concentrations such as Fe and Zn for bread wheat varieties (Morgounov et al. 2007; Oury et al. 2006; Wang et al. 2011) as well as for their wild and cultivated relatives (Chatzav et al. 2010; Gomez-Becerra et al. 2010; Peleg et al. 2008).

In maize, Menkir (2008) showed that there were highly significant effects of maize genotypes in mineral content, but the location effect was not significant in terms of the concentration of any kernel minerals, except Zn, in the majority of the trials. The mineral concentrations in maize grains can be affected by soil type and fertility, soil moisture, environmental factors, crop genotype and interactions among nutrients (Feila et al. 2005). Oikeh et al. (2003) reported that the effects of G × E were significant ($P < 0.05$) for grain Fe and Zn and was about double the contribution of the genotype (G) for grain Fe and Zn. However, G × E interaction can greatly influence genotypic performance across different crop-growing scenarios.

In common bean, results also indicate that the traits responsible for genetic improvements in Fe and Zn concentrations are stable across environments. Significant location and location × genotype effects indicate that environments have an influence on the concentrations of Fe and Zn in bean seeds. However, high-Fe and high-Zn accumulating genotypes will accumulate more nutrients when compared to low-Fe and low-Zn accumulating genotypes which were grown simultaneously at the same location, which once again shows that the environmental effect was absent and variation is purely due to the genotype. Interestingly, a very highly significant positive correlation of 0.52 between the concentrations of Fe and Zn across different genotypes were observed by CIAT researchers.

2.5 Genetic Association of Grain and Grain Yield in Micronutrient Concentration

Iron, zinc and copper are essential micronutrients for plants as well as humans (Asad and Rafique 2000; Hao et al. 2007). A deficiency of one of these nutrients can greatly reduce plant yield and even cause plant death. The correlation coefficients between Fe and Zn concentration and grain yield in cereal grain reported by earlier researchers are presented in Table 2.3. A recent study on micronutrient density in pearl millet showed no significant correlation between grain yield and Zn and Fe densities (Burger et al. 2014). Govindaraj et al. (2009) studied correlations between agro-morphological traits and densities of four minerals (P, Ca, Zn and Fe) in pearl millet, where no association with grain yields was observed for any of the four. However, studies on pearl millet, reported significant negative to no correlations between Zn (Fe) density and grain yield (Gupta et al. 2009; Rai et al. 2012; Velu et al. 2008). A negative correlation was observed between the

Table 2.3 Correlation coefficients between Fe and Zn concentrations and grain yield in cereal grains

Crop	Correlation coefficient (r)	References
Grain Fe and grain yield		
Bean	0.34*	Gelin et al. (2007)
Maize	−0.26*	Chakraborti et al. (2009)
Pearl millet	-0.02^{ns}	Gupta et al. (2009)
Sorghum	−0.32*	Reddy et al. (2005)
	−0.36*	Ashok Kumar et al. (2009)
Wheat	−0.39**	Vogel et al. (1989)
	−0.41*	Morgounov et al. (2007)
	-0.19^{ns}	Ficco et al. (2009) and Zhao et al. (2009)
	-0.51^{ns}	Oury et al. (2006)
Grain Zn and grain yield		
Bean	0.21*	Gelin et al. (2007)
Maize	0.18^{ns}	Chakraborti et al. (2009)
Pearl millet	-0.1^{ns}	Gupta et al. (2009)
Sorghum	−0.54**	Reddy et al. (2005)
	−0.46**	Ashok Kumar et al. (2009)
Wheat	−0.64**	Morgounov et al. (2007)
	−0.57 to −0.61**	McDonald et al. (2008)
	−0.41**	Ficco et al. (2009)
	−0.64**	Morgounov et al. (2007)
		Oury et al. (2006)
	−0.439**	Zhao et al. (2009)

*, ** = Significant at $P \leq 0.05$ and $P \leq 0.01$, respectively; *ns* non-significant

concentrations of Fe and Zn in grain and grain yield were reported in many studies in wheat, although the strength of these relationships was influenced greatly by the environment (White and Broadley 2009). There were obviously significant negative correlations between yield and Zn concentration with the correlation coefficients ranging from −0.67 to −0.41, while there was no significant correlation for Fe (Morgounov et al. 2007; Oury et al. 2006). In maize and sorghum, grain yield was found negatively associated with grain Fe (r = −0.26) and (r = −0.32 to −0.36), respectively. A low but positive correlation (r = 0.21) between grain yield and Zn and Fe have been reported in common bean. Grain yield and grain Zn were negatively associated in sorghum (r = −0.46 to −0.54). However, Anand et al. (2012) reported negative correlation between grain yield and mineral contents in rice. Grain Zn concentration showed negative correlation with grain yield per plant (r = −0.27) in recombinant inbred lines (RILs) of rice.

2.6 Heritability Estimates of Grain Iron and Zinc Concentrations

The inheritance of nutritional traits appears to be mostly quantitative, influenced by the environment, but more specific to source genotypes (Blair et al. 2009; Cichy et al. 2005, 2009). To determine whether Fe and Zn concentration in a particular crop can be improved by traditional breeding methods, it must be determined to what extent these traits are heritable. Heritability estimates are limited to experimental material and setup, and may differ widely in the same crop and for the same trait (Garcia-Oliveira et al. 2009). Heritability is a measure of genetic differences among individuals in a population, not simply of whether or not a trait is inherited (Gomez-Becerra et al. 2010). Heritability of Fe and Zn in the cited study was estimated by some researchers previously. Recently Govindaraj et al. (2011, 2013) and Bashir et al. (2013) reported high heritability estimates in pearl millet and suggested the predominance of additive gene effects in the inheritance of the nutritional traits. Both high heritability for grain Fe (65–71.2 %) and Zn (65–80 %) (Gupta et al. 2009) and heritability for grain Fe (80 %) and Zn (77 %) (Velu et al. 2007) have been reported in pearl millet, indicating that a substantial portion of the total variation for Fe/Zn is due to genetic effects. In wheat, estimates of broad-sense heritability (h2B) ranged from 90.62 % for Fe in 2010, to 90.90 % for Zn in 2011 (Badakhshan et al. 2013). Rawat et al. (2009) reported high heritability for grain Fe (0.98) and Zn (0.96) in wheat genotypes. Khodadadi et al. (2014) reported that the heritability of grain Fe and Zn in wheat was 0.74 and 0.61 in 2009 and 0.85 and 0.92 in 2010, respectively. Garcia-Oliveira et al. (2009) reported medium to high heritability for Fe and Zn, with estimates of 72.8 % and 40.6 %, respectively, in a set of recombinant inbred lines of rice. Chakraborti et al. (2010) reported high heritability for grain Fe (78 and 73 %) and grain Zn (71 and 76 %) in maize. Both moderate heritability (54 %) and high heritability (78–82 %) were reported for grain Zn in common bean (Cichy et al. 2005). Thus, heritability estimates are useful for the biofortification of high-yielding crop varieties.

2.7 Molecular Marker-Assisted Breeding for Genetic Improvement of Grain Fe and Zn Content in Crop Plants

The rapid development of DNA marker technology provides great opportunities to enhance nutritive values of traditionally-cultivated crops and grains. Molecular markers augment conventional plant breeding for efficient and precise identification or selection of a trait of interest linked to them. During the last few decades, molecular markers have been widely used in plant biotechnology and genetic studies. They are used in the assessment of genetic variability and characterization of

germplasm; estimation of genetic distance between populations, inbred and breeding material; genetic mapping; detection of monogenic and quantitative trait loci (QTLs); marker-assisted selection; increase in the speed and quality of backcrossing to introgress desirable traits from closely related varieties to elite germplasm and identification of sequences of useful candidate genes, etc. (Farooq and Azam 2002; Murtaza et al. 2005; Rana and Bhat 2005). Recent developments in quantitative genetics of molecular markers allow construction of linkage maps to determine the map position and effect of different loci/genes of metric characters i.e. QTLs. In QTL analysis, scientists attempt to identify associations between quantitative traits and marker alleles within a segregating population (Lander and Bostein 1989; Weller et al. 1990) to identify the genomic locations of loci contributing to complex traits, the contribution of each and the interaction between loci. QTL analysis provides a powerful approach to identify the genes underlying the natural variation for Fe and Zn concentrations (Ghandilyan et al. 2006). Molecular markers have been used to identify the genetic regions involved in grain Zn content in plants. Subsequently, there have been thousands of QTL studies carried out in different plant species.

In a study of wheat, nine additive and four epistatic QTLs were identified, among which six and four, respectively, were effective at the two environments (Xu et al. 2012). Peleg et al. (2009) found 11 QTLs on chromosomes 2A, 5A, 6B, 7A and 7B for Fe and 6 QTLs on chromosomes 2A, 2B, 3A, 4B, 5A, 6A, 6B, 7A and 7B for Zn. Shi et al. (2008) identified 4 QTLs for grain Zn concentration (mg/kg) on wheat chromosomes 4 and 5 contributing 11.9 % and 10.9 %, respectively, to the variance whereas for grain Zn content (μg/seed) seven major QTLs were found on chromosomes 2 and 7 in a double haploid wheat population. Genc et al. (2009) also reported major QTLs for grain Zn concentration on chromosomes 4 and 7 in wheat. A total of five significant QTLs controlling grain Zn and Fe content were detected in a maize $F_{2:3}$ mapping population (Jin et al. 2013). Lungaho et al. (2011) reported three modest QTLs for grain Fe concentration (FeGC) and ten QTLs for grain Fe bioavailability (FeGB) from an intermated B736Mo17 (IBM) recombinant inbred (RI) population of maize.

Identifying QTLs for Fe and Zn in rice grains, 14 QTLs were detected and QTLs for Fe were co-located with QTLs for Zn on chromosomes 7 and 12 (Anuradha et al. 2012). A total of seven QTLs for Fe and six for Zn were identified each explaining >30 % phenotypic variance in rice accessions (Neelamraju et al. 2012). Garcia-Oliveira et al. (2009) reported two QTLs for Fe on chromosomes 2 and 9 and three QTLs for Zn on chromosomes 5, 8 and 12. Three QTLs for Fe on chromosomes 2, 8 and 12, while two QTLs for Zn on chromosomes 1 and 12 and a common QTL for Fe and Zn accounted for a 13–14 % variation, as identified by Stangoulis et al. (2006). In common bean, a total of 26 QTLs were identified in an inter-gene pool mapping population for the mineral × trial × method combinations of which one-half were for Fe concentration and one-half for Zn concentration (Blair et al. 2009). Cichy et al. (2009) reported 11 QTLs on 6 linkage groups (LGs) accounting for 8–36 % variation for Fe and 11 QTL on 4 LGs accounting for 9–39 % variation in Zn.

However, marker-assisted selection is useful in improving the efficiency of selection early in the breeding cycle by helping to improve characters with low heritability. Thus, identifying the target QTL genes will help achieve biofortification with greater precision and accuracy.

2.8 Transgenic Approaches for Micronutrient Improvement

Transgenic approaches are advantageous when a micronutrient does not naturally exist in a crop (e.g. provitamin A in rice) or when sufficient amounts of bioavailable micronutrients cannot be effectively bred into the crop. However, once a transgenic line is obtained, several years of conventional breeding are needed to ensure that the transgenes are stably inherited and to incorporate the transgenic line into varieties that farmers prefer. While transgenic breeding can sometimes offer micronutrient gains beyond those available to conventional breeders, many countries lack the legal framework to allow release and commercialization of these varieties. To attain higher levels of provitamin A, Zn and Fe content in crops where genetic variation for these traits has not been identified, HarvestPlus, its partners, and other organizations have explored transgenic approaches, discussed below in detail.

2.8.1 Golden Rice

Golden Rice is a variety of *Oryza sativa* produced through genetic engineering to biosynthesize beta-carotene, a precursor of vitamin A, in the edible parts of rice (Ye et al. 2000). It was first developed at the Swiss Federal Institute of Technology and the University of Freiburg, Germany. Golden Rice was created by transforming rice with only two beta-carotene biosynthesis genes: psy (phytoene synthase) from daffodil (*Narcissus pseudonarcissus*) and crtI (carotene desaturase) from the soil bacterium *Erwinia uredovora* (Fig. 2.3).

In 2005, a research team at the Syngenta biotechnology company produced a variety of Golden Rice called Golden Rice 2. It combined the phytoene synthase gene from maize with crt1 from the original Golden Rice. Golden Rice 2 produces 23 times more carotenoids than the original Golden Rice (up to 37 μg/g), and preferentially accumulates beta-carotene (up to 31 μg/g of the 37 μg/g of carotenoids) (Paine et al. 2005). To receive the Recommended Dietary Allowance (RDA), it is estimated that 144 g of the highest-yielding strain would have to be eaten. Bioavailability of the carotene from Golden Rice has been confirmed and found to be an effective source of Vitamin A for humans (Datta et al. 2007; Tang et al. 2009). Bioavailability testing has confirmed that Golden Rice is an effective source of vitamin A in humans, with an estimated conversion rate of beta-carotene to retinol of 3.8:1 and 2:1 (Tang et al. 2009, 2012).

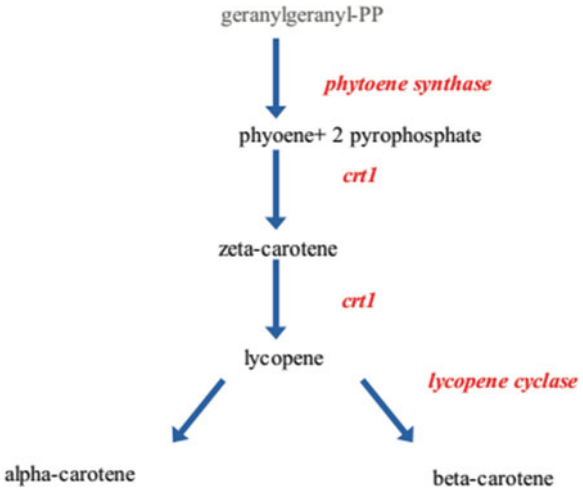

Fig. 2.3 A simplified overview of the carotenoid biosynthesis pathway in Golden Rice. The enzymes expressed in the endosperm of Golden Rice, shown in red, catalyze the biosynthesis of beta-carotene from geranylgeranyl diphosphate. Beta-carotene is assumed to be converted to retinal and subsequently retinol (vitamin A) in the animal gut (Source: http://en.wikipedia.org/wiki/Golden_rice)

2.8.2 Iron-Rich Rice

Iron deficiency is considered one of the world's most widespread micronutrient deficiencies. Despite the fact that whole grains, vegetables and fruits contain Fe, absorption of the micronutrient is poor from these food sources because it is bonded with phytic acid. Since rice is a staple food for over three billion people, improving its Fe content (normal availability of Fe 0.2–2.8 mg/100 g rice) could help resolve the problem of Fe deficiency especially in developing countries. Researchers have incorporated pAGt lFe containing the gene for the ferritin protein from *Phaseolus vulgaris* and pAGt 1Me with metallothirnein-like protein followed by agrobacterium-mediated transformation, which increased the Fe content in the rice endosperm twofold (Lucca et al. 2002). To address the bioavailability problem, Lucca et al. (2002) integrated the gene from *Aspergillus fumigatus* encoding a thermotolerant phytase protein and the gene for endogenous cysteine-rich metallothionein-like protein. Cysteine helps increase Fe uptake during digestion. The concerted effect of these genes resulted in a sevenfold increase in cysteine level and a 130-fold increase in phytase level. Masuda et al. (2013) recently reported seven transgenic approaches to increase the Fe concentration of rice seeds (Tables 2.4 and 2.5) and also proposed some additional prospective target genes for the Fe biofortification of rice.

Table 2.4 Approaches of Fe biofortification of rice: single transgenic approaches

Approach cultivation	Introduced genes	Rice cultivar	Cultivation condition	Fold increase in Fe concentration compared to non-transgenic rice[a]	References
Approach 1: enhancement of Fe storage in rice seeds by ferritin	OsGluB1pro-SoyferH1	Japonica cv. Kitaake	Soil cultivation in greenhouse	2 fold (polished seeds)	Goto et al. (1999)
	OsGluB1 pro-SoyferH1[b]	Japonica cv. Kitaake	Soil cultivation in greenhouse	3 fold (brown seeds)	Qu et al. (2005)
	OsGlb1 pro-SoyferH1b			1.5 fold (brown seeds)	
	OsGluB1 pro-SoyferH1	Japonica cv. Taipei 309	Soil cultivation in greenhouse	2.2 fold (brown seeds)	Lucca et al. (2002)
	OsGluB1 pro-SoyferH1	Indica cv. IR68144	Soil cultivation in greenhouse	3.7 fold (polished seeds)	Vasconcelos et al. (2003)
	OsGluA2 pro-OsFer2	Indica cv. Pusa-Sugandh II (aromatic rice)	Soil cultivation in greenhouse	2.1 fold (polished seeds)	Paul et al. (2012)
Approach 2: enhancement of Fe translocation by overexpression of NAS	OsActin1 pro-HvNAS1c	Japonica cv. Tsukinohikari	Soil cultivation in greenhouse	2 fold (polished seeds)	Masuda et al. (2009)
	35S pro-HvNAS1c				
	Activation tag line of OsNAS3	Japonica cv. Dongjin	Soil culture in greenhouse	3 fold (polished seeds)	Lee et al. (2009c)
	35S pro-OsNAS1, 2, 3	Japonica cv. Nipponbare	Soil cultivation in greenhouse	4 fold (polished seeds)	Johnson et al. (2011)
Approach 3: enhancement of Fe transportation by Fe transporter	OsSUT1 pro-OsYSL2	Japonica cv. Tsukinohikari	Soil cultivation in greenhouse	4 fold (polished seeds)	Ishimaru et al. (2010)

(continued)

Table 2.4 (continued)

Approach cultivation	Introduced genes	Rice cultivar	Cultivation condition	Fold increase in Fe concentration compared to non-transgenic rice[a]	References
Approach 4: enhancement of Fe uptake and translocation by IDS3 gene	Barley IDS3 genome fragment	Japonica cv. Tsukinohikari	Andosol soil in paddy field	1.4 fold (polished seeds)	Masuda et al. (2008)
				1.3 fold (brown seeds)	
			Calcareous soil in paddy field	1.3 fold (brown seeds)	Suzuki et al. (2008)
Approach 5: overexpression of Fe transporter	Ubiquitin pro-OsIRT1	Japonica cv. Dongjin	Paddy field	1.7fold (leaves)	Lee et al. (2009a)
				1.1 fold (brown seeds)	
	OsActin1 pro-OsYSL15	Japonica cv. Dongjin	Paddy field	1.3 fold (brown seeds)	Lee et al. (2009b)
Approach 6: overexpression of transcription factor	35S pro-OsIRO2	Japonica cv. Tsukinohikari	Calcareous soil in greenhouse	3 fold (brown seeds)	Ogo et al. (2011)
Approach 7: knockdown of OsVITs genes	OsVIT1 or OsVIT2 T-DNA insertion mutant lines	Japonica cv. Zhonghua11	Hydroponic culture	1.4 fold (brown seeds)	Zhang et al. (2012)
		Japonica cv. Dongjin	Paddy field	1.4 fold (brown seeds)	
	OsVIT2 T-DNA insertion mutant line	Japonica cv. Dongjin	Soil cultivation in greenhouse	1.3 fold (brown seeds)	Bashir et al. (2013)
				1.8 fold (polished seeds)	

Source: Masuda et al. (2013)

[a]The tissue name in parentheses is the rice tissue where Fe concentration was increased

[b]They introduced these two genes into same transgenic lines

[c]These two genes were introduced separately into rice and they analyzed these two types of transgenic lines

Table 2.5 Approaches of Fe biofortification of rice: multi-transgenic approaches

Approach cultivation	Introduced genes[a]	Rice cultivar	Cultivation condition	Fold increase in Fe concentration compared to non-transgenic rice[b]	References
Combination of approaches 1 and 2	OsGlb pro-Pvferritin	Japonica cv. Taipei 309	Hydroponic culture	6 fold (polished seeds)	Wirth et al. (2009)
	35S pro-AtNAS1				
	OsGlb pro-Afphytase				
Combination of approaches 1, 2 and 3	OsGluB1 pro-SoyferH2	Japonica cv. Tsukinohikari	Soil cultivation in greenhouse	6 fold (polished seeds)	Masuda et al. (2012)
	OsGlb1 pro-SoyferH2				
	OsActin1 pro-HvNAS1		Paddy field	4.4 fold (polished seeds)	
	OsSUT1 pro-OsYSL2				
	OsGlb1 pro-OsYSL2				
	OsGluB1pro-SoyferH2	Tropical Japonica cv. Paw San Yin (Myanmar high quality rice)	Soil cultivation in greenhouse	3.4 fold (polished seeds)	Aung et al. (2013)
	OsGlb1 pro-SoyferH2				
	OsActin1pro-HvNAS1				
	OsSUT1 pro-OsYSL2				
	OsGlb1 pro-OsYSL2				
Combination of approaches 1 and 4	OsGluB1 pro-SoyferH2	Japonica cv. Tsukinohikari	Normal soil in greenhouse	4 fold (polished seeds)	Masuda et al. (2013)
	OsGlb1 pro-SoyferH2				
	HvNAS1, HvNAAT-A,-B and IDS3 genome fragments		Calcareous soil in greenhouse	2.5 fold (polished seeds)	

Source: Masuda et al. (2013)

[a]These gene expression cassettes were introduced concomitantly

[b]The tissue name in parentheses is the rice tissue where Fe concentration was increased

2.9 Micronutrient Bioavailability

The total amount of a micronutrient from a plant source does not represent the actual micronutrient content of the food that is utilizable by the consumer. The bioavailability of micronutrients must be determined independently using methodologies especially developed for such purposes. In human nutrition terms, bioavailability is commonly defined as the amount of a nutrients in a meal that is absorbable and utilizable for metabolic processes in the body (Welch and Graham 2004). Determining the bioavailability of micronutrients to humans in plant foods is fraught with difficulty (Fig. 2.4). Ultimately to determine the bioavailability of a particular micronutrient a number of factors interact in the body of an individual eating a mixed diet within a given environment. Because of this complexity, the data obtained using various bioavailability model systems are always ambiguous (House 1999; Van Campen and Glahn 1999).

Not all ingested minerals are completely absorbed and utilized by humans or livestock (Grusak and Cakmak 2004); moreover, only a small portion of accumulated minerals in edible parts is bioavailable leading to certain groups of people who are vegetarians being at risk of deficiencies of Fe, Zn and other trace elements. Thus, determining the bioavailability of Fe and Zn in genetically-enhanced new lines is an important aspect of a crop biofortification program. The levels of bioavailable Fe and Zn in staple food crop grains are as low as 5 % and 25 %, respectively (Bouis and Welch 2010). Researchers should therefore consider the bioavailability of micronutrients and their concentration while conducting breeding experiments.

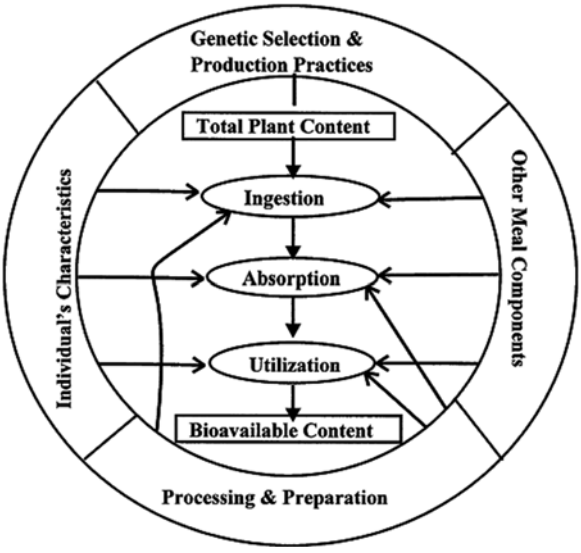

Fig. 2.4 The complexities of bioavailability in human nutrition (Source: Graham et al. 2001)

Only data from feeding trials in micronutrient-deficient test populations under free-living conditions can delineate the efficacy of using micronutrient-enriched varieties of plant foods as an intervention tool. Unfortunately, it is impractical to test the bioavailability of selected micronutrients in numerous genotypes of staple plant foods that can be generated in plant breeding programs (Graham and Welch 1996). Therefore, to screen large numbers of promising lines of micronutrient-enriched genotypes identified through a breeding program one must use a bioavailability model before advancing them within these programs.

2.9.1 Bioavailability Models

Various bioavailability models have been developed to determine the micronutrients in human plant foods (House 1999; Van Campen and Glahn 1999). Among these in wide use are in vitro models such as cultured human intestinal cells (i.e. Caco-2 cell model), animal models (e.g. rats, pigs and poultry) and small-scale human clinical trials (Underwood and Smitasiri 1999). The rat and poultry models are easy to execute and relatively cost effective, but the results obtained are limited in their acceptance by the nutrition community (Gibson et al. 1994). In vitro cultured human intestinal cell models such as the Caco-2 cell model are rapid, inexpensive and can be used to screen large numbers of genotypes for bioavailable Fe (Van Campen and Glahn 1999). However, the Caco-2 cell model needs further development before adopting it to determine the bioavailability of Zn and provitamin A carotenoids in staple plant foods. The pig animal model is currently and widely accepted, as it is the most accurate of the animal models available to study the bioavailability of Fe, Zn and provitamin A carotenoids in plant foods (Miller and Ullrey 1987). Current breeding efforts to screen large numbers of promising genotypes rich in micronutrients of staple foods crops (rice, maize, pearl millet, sorghum, wheat, beans and manioc) at several CGIAR Centers (IRRI, CIMMYT, ICRISAT, CIAT and IITA) for bioavailable Fe, rely on an in vitro Caco-2 cell model.

Bioavailability of Fe and Zn is known to be influenced by various dietary components, which include both absorption inhibitors and enhancers. Among the inhibitors, phytic acid (PA), tannins, dietary fiber and calcium are the most potent, while organic acids are known to promote Fe absorption (Elad et al. 2015; Gibson et al. 1994; Hambidge et al. 2010; Sandberg and Andlid 2002). Phytate, a complex of phytic acid and mineral elements, decreases the bioavailable concentration of nutrient elements and thus leads to health problems, such as Fe and Zn deficiency, in populations with diets based mainly on cereals and legumes (Liu et al. 2006). These compounds are normal plant metabolites and only small changes in their concentration may have significant effects on the bioavailability of micronutrients.

Several studies have demonstrated the negative effect of phytate on Zn and Fe absorption, causing nutritional deficiencies both in humans and livestock (Lonnerdal 2000). A study of pearl millet showed that Fe was chelated by phytates and insoluble fibers, whereas Zn was almost exclusively chelated by phytates. A recent study

on high Fe pearl millet by Tako et al. (2015) showed that higher-Fe pearl millet provides more absorbable Fe that is limited by increased polyphenolic content. Similarly, in the case of higher fiber and tannin contents, the chelating effect of these compounds was higher than that of phytates (Lestienne et al. 2005). Results of pilot studies among maize consumers in the USA and Guatemala showed that genetically-selected low phytic acid plants have the potential to be used as primary or complementary strategies in the prevention of human Zn deficiency (Hambidge et al. 2004). Studies in animals have shown the positive effect of diets containing low phytate maize to improve the use of minerals (Li et al. 2000; Veum et al. 2001). Therefore, food crop breeding strategies for higher levels of nutrients and low levels of anti-nutritional substances, such as phytic acid, are desirable (Ghandilyan et al. 2006). Thus, the inhibitory effect of phytate should be taken into account when assessing Fe and Zn deficiencies.

Recent technological advancements have improved the accuracy and precision of methods used in the study of bioavailability and absorption of trace elements. Currently two models are used to evaluate mineral bioavailability in foods and diets, each giving a great variability of results: in vivo and in vitro models (Vitali et al. 2007; Welch and Graham 2002). In vivo investigations generally include work with rats or clinical studies with humans. In vitro methods involve determining the soluble and/or dialyzable fraction of the mineral and are important as screening techniques (Fairweather-Tait et al. 1995). Due to the phytic acid influence on mineral absorption, researchers have also used the molar ratio of phytic acid/mineral as a simpler and less costly method to estimate the Fe and Zn bioavailability in food (Abebe et al. 2007; Lestienne et al. 2005). In vivo and in vitro studies on the availability of Fe in a nutritional formulation indicated low Fe availability and absorption in humans (Bueno et al. 2013).

2.10 Biofortification: A Tool for Improved Human Health

Breeding staple cereal crops richer in minerals is a low-cost, sustainable strategy to ameliorate micronutrient malnutrition for people living in developing countries who cannot afford to include sufficient amounts of pulses, fruits, vegetables, fish and animal products, rich or enriched with micronutrients in their diet (Cakmak 2008; Martinez et al. 2010). A combination of strategies involving food fortification, pharmaceutical supplementation and dietary diversification has been suggested to combat micronutrient malnutrition (Stein et al. 2005). However, neither strategy has been universally successful in developing countries, largely due to lack of safe delivery systems, stable government policies, appropriate infrastructure and continued adequate investment (Bouis 2003; Timmer 2003). Thus, biofortification has been proposed as an alternative solution to micronutrient malnutrition (Bouis 2003). Biofortification is a new approach to combat micronutrient deficiencies, by increasing the concentration and/or bioavailability of essential elements in the edible part of the plant by traditional plant breeding or genetic engineering (White and Broadley 2005). By definition, the focus of plant

breeders and biofortification initiatives is on breeding crops with a high density and increased bioavailability of nutrients. HarvestPlus (www.harvestplus.org) is a major international consortium created to develop new plant genotypes with high concentrations of micronutrients by applying classical and modern breeding tools (i.e. genetic biofortification). Although plant breeding is the most sustainable solution to the problem, developing new micronutrient-rich plant genotypes is a protracted process and its effectiveness can be limited by the low amount of readily-available pools of soluble micronutrients in soils (Cakmak 2008). Application of fertilizers containing Zn and Fe (i.e. agronomic biofortification) is a short-term solution and represents a complementary approach to breeding. Biofortified crops, once developed, adapted and released for cultivation, will continue to be grown and consumed yearly, thus contributing significantly to overcoming malnutrition (Graham et al. 2007; Stein et al. 2005; Stein 2010; White and Broadley 2009). Recent studies report clear increases in Fe and Zn absorption when biofortified pearl millet grain of Indian origin is consumed by young women or children (Cercamondi et al. 2013; Kodkany et al. 2013). Another study showed strong positive correlation ($r=0.73$) between Zn and Fe, showing that the simultaneous selection for high Zn and Fe densities could be very efficient (Burger et al. 2014; Kanatti et al. 2014). Several studies reported a high correlation between Zn and Fe in pearl millet (Bashir et al. 2013; Govindaraj et al. 2009; Velu et al. 2007) and in wheat (Gomez et al. 2010; Velu et al. 2012). In wheat, Fe and Zn correlate positively and the highest concentrations (up to 85 μg/g) were detected in landraces as well as in wild and primitive relatives (Ortiz-Monasterio et al. 2007; Peleg et al. 2009). In India, application of Zn-coated urea fertilizer significantly improved both grain yield and grain Zn concentrations (Shivay et al. 2008).

Conventional plant breeding and genetic engineering both involve changing the genotype of targeted crops with the aim of developing plants carrying genes that support the enhanced accumulation of bioavailable minerals. The means of achieving this goal differ between the two approaches (Gomez-Galera et al. 2010). The main nutrients targeted for biofortification are beta carotene, Fe and Zn. Most current research is being done on traditional plant breeding techniques, exploiting the variability of mineral concentrations found in different germplasm (Qaim et al. 2007). Not all crops have the genetic potential to meet desired micronutrient levels with traditional plant breeding, and therefore genetic engineering has to be applied to achieve sufficient improvements (Borg et al. 2009). It is suggested that genetic modification is an excellent approach to obtain high micronutrient concentrations (Bouis 2007) and that genetically-modified organisms (GMOs) have the potential for increased agricultural productivity.

Another genetic engineering approach to increasing the bioavailability of Fe in diets is the elimination of phytate. This sugar-like molecule binds a high proportion of dietary Fe, so that the human body is unable to absorb it. Lucca et al. (2001) introduced a fungal gene for the enzyme phytase, which breaks down phytate synthesis, thus improving the bioavailability of Fe in rice diets. Wei et al. (2012) reported that foliar Zn fertilization reduced the phytic acid content and increased the accumulation of bioavailable Zn in polished rice. In maize, overexpression of *Aspergillus niger* phytase gene (phyA2) in seeds using a construct driven by the maize embryo-specific

globulin-1 promoter resulted in about 5,000 % increase in phytase activity and 30 % decrease in seed phytate concentration. On the other hand, a very novel and interesting approach has been used in maize and soybean to silence the genes involved in the biosynthesis of phytic acid (PA) (Shi et al. 2008). It was found that maize lpa1 mutants are defective in a MRP ATP-binding cassette (ABC) transporter that is more highly expressed in embryos, but also in immature endosperm, germinating seeds and vegetative tissues. The expression of this transporter was silenced in an embryo-specific manner. The concentration of PA in seeds of transgenic maize was found to be reduced by up to 87 % depending upon the transgenic line, and the transgenic plants were not adversely affected in grain yield or seed germination in contrast to the lpa mutants. Similarly, silencing of MRP (expansion) transporter in sorghum decreased the PA concentration in seeds by 80–86 %, and a consequent increase in Fe and Zn absorption was observed when analyzed in Caco-2 cell lines (Kruger et al. 2013). These remarkable findings indicate the possibility of producing GMO cereals with low PA and without affecting agronomic performance by silencing the expression of transporters involved in the biosynthesis of PA.

2.11 Conclusion and Prospects

Biofortification is a method of breeding crops to increase their nutritional value. This can be done either through conventional selective breeding or through genetic engineering. Biofortification differs from ordinary fortification because it focuses on making plant foods more nutritious as they are growing, rather than having nutrients added to processed foods. This is an improvement over ordinary fortification when it comes to providing nutrients for the rural poor, who rarely have access to commercially-fortified foods. As such, biofortification is seen as a future strategy to deal with deficiencies of micronutrients in the developing world. In the case of Fe, WHO estimated that biofortification could help cure the two billion people suffering from iron deficiency-induced anemia.

There is very compelling global human health and nutritional evidence to convince plant breeders that micronutrient density traits should be primary objectives in their work, and targeted to the developing world. Therefore, biofortification is of great importance in enriching seeds with mineral micronutrient. Both plant breeding and genetic modification offer good opportunities to increase the micronutrient contents of edible parts of major crops. Anti-nutrient factors should be minimized to maximize micronutrient bioavailability. Understanding the genetic basis for breeding crop cultivars with higher grain micronutrient concentration is required. Emerging cost-effective genomics tools should be used to accelerate the breeding process and product development targeting these micronutrients. After development of new breeding lines and varieties, dissemination of biofortified breeding lines and hybrid parents to and their utilization by user-research organizations in the public and private sector on a continuing basis will make biofortified cultivar development a routine matter and significantly contribute to improved human nutrition.

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Chapter 3
Role of Genomics in Enhancing Nutrition Content of Cereals

Mehanathan Muthamilarasan and Manoj Prasad

Abstract Unprecedented growth of global population along with increasing levels of malnutrition among infants and adults necessitate the immediate research on improving the nutritional quality of major crops. Although sufficient research is in progress towards generating elite varieties capable of withstanding adverse climates without affecting their productivity, less importance has been given to ensure the nutritional properties of these crops. Cereals constitute a major source of staple food to the global population, but the levels of micro- and macro-nutrients in cereals are considerably less when compared to millets. Millets serve as versatile crops with exceptional agronomic traits as well as nutritional characteristics. Unlike cereals, millets are C_4 photosynthetic crops with several salient features such as tolerance to broad-spectrum abiotic stresses, adaptation to a wide range of ecological conditions, better survival and productivity in nutrient poor soils and are nutritionally superior to major cereals. In this context, this chapter summarizes the advancements made in the area of crop genomics with emphasis on improvement of the nutritional content of millets. Considering the genetically close-relatedness between millets and cereals, research should focus on investigating the genetics and genomics of nutritional traits in millets and introgress them into cereals using integrated *omics* approaches.

Keywords Nutrition • Genomics • Millets • Next generation sequencing • Cereals • Biofortification

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3.1 Introduction

Climate change poses a serious threat to future agriculture and farming, and predictions of the Intergovernmental Panel on Climate Change (IPCC) that the global temperature is anticipated to increase, further challenges food security. The changes on the arable lands brought about by the increase in temperatures are now apparent and it has been reported that millions of hectares of agricultural area have become barren. Along with this, malnutrition and hunger among the world population are at alarming rates, which adds up to global food insecurity. Furthermore, the FAO Hunger Report (2012) depicts that, about 12.5 % of the global population (one in eight people) is starving, excluding 100 million children under the age of 5. Irrespective of the adults, about 2.5 million children die every year due to hunger and malnutrition which ultimately hinders human potential (FAO Hunger Report 2012).

Since plants are the primary producers in the food chain, they serve as versatile biochemical factories capable of producing almost a complete complement of essential dietary micronutrients. However, the dietary micronutrients are unevenly disseminated among different plant parts. For instance, the iron content in a rice leaf is as high as 100–200 ppm, but very low in the polished rice grain (~3 ppm) (Mayer et al. 2008). Similarly, provitamin A carotenoids are present only in rice leaves but not in its edible part. Unfortunately, economically-backward people rely predominantly on starchy staples such as rice, wheat, maize or cassava, but these crops do not supplement the biochemical diversity needed for a healthy life, which leads to micronutrient malnutrition (MNM). Plant science has a central role in addressing these issues of both hunger and malnutrition. Since, MNM affects more than half of the world population, biotechnology-assisted nutrition-enhancement offers an economical and sustainable approach to deliver nutrients via nutrient-dense crops to the human population. Hence, this chapter summarizes the strategies of generating nutrition-rich crop plants through biotechnological advances with emphasis on genomic strategies. In addition, it summarizes the accomplishments reported in enhancing the nutrition content through non-genomic based approaches in major cereals and demonstrates how genomics-assisted nutrition enhancement would be a better strategy for effectively achieving this in a short-span of time using nutrition-rich foxtail millet as a model.

3.2 Progress in Crop Genome Sequencing and Analysis

Advancements in next-generation sequencing (NGS) and high-throughput analysis platforms have made possible whole genome sequencing of many model and non-model crops. So far, more than 50 plant genomes have been sequenced of which 8 are models (Table 3.1). This sequencing information is available to the global scientific community through open-access databases and the information would be useful in deciphering the biological and molecular roles of individual genes and their

Table 3.1 List of sequenced plant genomes

Scientific name	Common name	Year of sequencing	Type	Division	Chr.	Genome size (Mb)	Genes	Repeat percentage	References
Aegilops tauschii	Wheat DD	2013	Crop	Monocot	7	4,360	43,150	66	Jia et al. (2013)
Arabidopsis lyrata	Lyrata	2011	Model	Dicot	8	207	32,670	30	Hu et al. (2011)
A. thaliana	Arabidopsis	2000	Model	Dicot	5	125	25,498	14	AGI (2000)
Azadirachta indica	Neem	2012	Crop	Dicot	Na	364	20,169	13	Krishnan et al. (2012)
Brachypodium distachyon	Brachypodium	2010	Model	Monocot	5	272	25,532	21	IBI (2010)
Brassica rapa	Chinese cabbage	2011	Crop	Dicot	10	485	41,174	40	Wang et al. (2011)
Cajanus cajan	Pigeon pea	2011	Crop	Dicot	11	833	48,680	52	Varshney et al. (2011)
Cannabis sativa	Hemp	2011	Crop	Dicot	Na	820	30,074	Na	van Bakel et al. (2011)
Capsella rubella	Capsella	2013	Nonmodel	Dicot	8	219	26,521	Na	Slotte et al. (2013)
Capsicum annuum	Pepper	2014	Crop	Dicot	12	3,480	34,903	81	Kim et al. (2014)
Carica papaya	Papaya	2008	Crop	Dicot	9	372	28,629	43	Ming et al. (2008)
Cicer arietinum	Chickpea	2013	Crop	Dicot	8	738	28,269	49	Varshney et al. (2013)
Citrullus lanatus	Watermelon	2012	Crop	Dicot	11	425	23,440	45	Guo et al. (2012)
Cucumis melo	Melon	2012	Crop	Dicot	12	450	27,427	NA	Garcia-Mas et al. (2012)

(continued)

Table 3.1 (continued)

Scientific name	Common name	Year of sequencing	Type	Division	Chr.	Genome size (Mb)	Genes	Repeat percentage	References
C. sativus	Cucumber	2009	Crop	Dicot	7	367	26,682	24	Huang et al. (2009)
C. sativus	Cucumber	2011	Crop	Dicot	7	367	26,587	Na	Wóycicki et al. (2011)
Fragaria vesca	Strawberry	2011	Crop	Dicot	7	240	34,809	23	Shulaev et al. (2011)
Glycine max	Soybean	2010	Crop	Dicot	20	1,115	46,430	57	Schmutz et al. (2010)
Gossypium raimondii	Cotton	2012	Crop	Dicot	13	880	40,976	60	Patterson et al. (2012)
G. raimondii	Cotton D	2012	Crop	Dicot	13	880	37,505	61	Wang et al. (2012a)
Hevea brasiliensis	Rubber tree	2013	Crop	Dicot	18	2,150	68,955	72	Rahman et al. (2013)
Hordeum vulgare	Barely	2012	Crop	Monocot	7	5,100	30,400	84	IBGSC (2012)
Jatropha curcas	Jatropha	2010	Crop	Dicot	Na	380	40,929	37	Sato et al. (2011)
Linum usitatissimum	Flax	2012	Crop	Dicot	15	373	43,484	24	Wang et al. (2012b)
Lotus japonicus	Lotus	2008	Model	Dicot	6	472	30,799	56	Sato et al. (2008)
Malus x domestica	Apple	2010	Crop	Dicot	17	742	57,386	67	Velasco et al. (2010)
Medicago truncatula	Medicago	2011	Model	Dicot	8	454	62,388	31	Young et al. (2011)
Musa acuminate malaccensis	Banana	2012	Crop	Monocot	11	523	36,542	44	D'Hont et al. (2012)

Nelumbo nucifera	Ancient lotus	2013	Nonmodel	Dicot	8	929	26,685	57	Ming et al. (2013)
Oryza brachyantha	Rice relative	2013	Nonmodel	Monocot	12	300	32,038	29	Chen et al. (2013)
O. sativa	Rice	2002	Crop	Monocot	12	430	59,855	26	Yu et al. (2002)
O. sativa	Rice	2002	Crop	Monocot	12	420	61,668	Na	Goff et al. (2002)
Phoenix dactylifera	Date palm	2011	Crop	Monocot	18	658	28,890	40	Al-Mssallem et al. (2011)
Phyllostachys heterocycla	Moso bamboo	2013	Nonmodel	Monocot	24	2,075	31,987	51	Peng et al. (2013)
Physcomitrella patens	Moss	2008	Model	Bryophyta	27	510	35,938	16	Rensing et al. (2008)
Picea abies	Norway spruce	2013	Crop	Gymnosperm	12	19,600	28,354	Na	Nystedt et al. (2013)
Populus trichocarpa	Poplar	2006	Crop	Dicot	19	485	45,555	Na	Tuskan et al. (2006)
Prunus mume	Chinese plum	2012	Crop	Dicot	8	280	31,390	45	Zhang et al. (2012b)
P. persica	Peach	2013	Crop	Dicot	8	265	27,852	37	IPGI (2013)
Pyrus bretschneideri	Pear	2013	Crop	Dicot	17	527	42,812	53	Wu et al. (2013)
Ricinus communis	Castor bean	2010	Crop	Dicot	10	320	31,237	50	Chan et al. (2010)
Selaginella moellendorffii	Spikemoss	2011	Nonmodel	Lycopod	Na	110	22,285	38	Banks et al. (2011)
Setaria italica	Foxtail millet	2012	Model	Monocot	9	490	38,801	46	Bennetzen et al. (2012)
Solanum lycopersicum	Tomato	2012	Crop	Dicot	12	900	34,727	63	TGC (2012)
S. tuberosum	Potato	2011	Crop	Dicot	12	844	39,031	62	PGSC (2011)

(continued)

Table 3.1 (continued)

Scientific name	Common name	Year of sequencing	Type	Division	Chr.	Genome size (Mb)	Genes	Repeat percentage	References
Sorghum bicolor	Sorghum	2009	Crop	Monocot	10	818	34,496	62	Paterson et al. (2009)
Thellungiella parvula	Thellungiella	2011	Model	Dicot	7	140	30,419	8	Dassanayake et al. (2011)
Theobroma cacao	Cocoa	2011	Crop	Dicot	10	430	28,798	24	Argout et al. (2011)
Triticum aestivum	Wheat	2012	Crop	Monocot	21	17,000	94,000	80	Brenchley et al. (2012)
T. aestivum	Wheat	2014	Crop	Monocot	21	17,000	124,201	80	IWGSC (2014)
T. urartu	Wheat AA	2013	Crop	Monocot	7	4,940	34,879	67	Ling et al. (2013)
Utricularia gibba	Bladderwort	2013	Nonmodel	Dicot	16	77	28,500	3	Ibarra-Laclette et al. (2013)
Vitis vinifera	Grape	2007	Crop	Dicot	19	475	30,434	41	Jaillon et al. (2007)
V. vinifera	Grape	2007	Crop	Dicot	19	505	29,585	27	Velasco et al. (2007)
Zea mays	Maize	2009	Crop	Monocot	10	2,300	32,540	85	Schnable et al. (2009)

NA Not available, *Chr.* Chromosome

participation in regulatory networks. Furthermore, genome sequence data would also assist in understanding evolutionary relationships and to explore several unidentified regulatory mechanisms which work in harmony for better yield and stress tolerance in crop plants (Muthamilarasan et al. 2013a). Although genomics-based approaches have been well developed in animal sciences, crop genomics has started gaining equal importance for providing healthy food and feed for a growing population. The drastic increase in global population along with increased malnourished and undernourished infants and adults compels the effective use of these genome data for generating elite varieties with better products. In addition to whole genome sequencing, transcriptome sequencing has also been performed to identify and analyze expressed genes (mRNAs). In contrast to whole genome sequencing, transcriptome sequencing has enabled researchers to identify differentially-expressed genes between any contrasting cultivars, time points, related species or tissues. Furthermore, transcriptome sequencing also facilitates the analysis of expression levels of transcripts.

The whole genome sequence could be a standard draft, high-quality draft, improved high-quality draft, non-contiguous finished, finished and gold-standard sequence (Feuillet et al. 2011). The standard draft serves as a base for identification and cataloging of genes and repeat elements in a genome. Furthermore, it also assists in understanding the evolution and performing syntenic analyses using comparative mapping and phylogenetic analysis. The high-quality draft is considerably better than the standard draft as it enables studying lineage-specific features. But the prime drawbacks of draft and high-quality draft sequences are the difficulties in identification of pseudogenes and recent segmental duplications. Therefore, full annotation and functional analysis of genomes are required which is provided by improved a high-quality draft. This sequence information would also assist in epigenomic and epigenetic studies. For identifying variations underlying the phenotypes and performing lineage specific biology, non-contiguous finished, finished genomes are necessary, whereas gold-standard sequence information expedites cloning, imparting stress tolerance and understanding the complete biology. Crops which serve as models for improving other important plant species have been given priority for whole genome sequencing. This enables the translation biology and, especially, identifying the specific-specific differences which underlie important traits. With this summary on genome sequencing, the following sections explain how these data could be effectively used in enhancing nutrition levels in crop plants.

3.3 Approaches for Generating Nutrient-Rich Crops

3.3.1 Conventional Plant Breeding

Traditional plant breeding is being used predominantly for improving crop yields and enhancing crop resistance to environmental stresses, but several studies have supported the implementation of breeding for enhancing nutrient content.

Unfortunately, reports have revealed that the increase in productivity over the last four decades has been accompanied by a reduction in concentration of minerals including Fe, Zn, Cu and Mg in edible plant tissues (Fan et al. 2008; Garvin et al. 2006; White and Broadley 2009). Since conventional plant breeding discovers the inherent characteristics of the diverse crop varieties, this strategy for improving the nutrient content received widespread public acceptance and a simple legal framework (Bouis 2000; Hirschi 2009). Moreover, this nutrition-improvement strategy only represents a one-off cost as it involves a single initial subsidized distribution and the seeds can be harvested and used in future years (Carvalho and Vasconcelos 2013). These merits make this cost-effective and long-term nutrition-improvement strategy as the most expedient solution for improving the micronutrient density of edible plant tissues (Hirschi 2009). On the other hand, this strategy also encompasses certain disadvantages, such as: (1) long development time, (2) dependence on the phytoavailability of the mineral nutrients in the soil and (3) need for sufficient genetic variation of a given trait within species. However, many traits needed in the nutrition-improvement approach can be identified by exploring the genetic variation in germplasm collections or by exploiting transgressive segregation or heterosis (Carvalho and Vasconcelos 2013; Mayer et al. 2008).

Numerous studies have reported the existence of large within-species genetic variation in various crops, both in terms of the concentration of nutrients in the edible tissues and their bioavailability to the human gastric system (White and Broadley 2009). For example, various rice genotypes exhibit a fourfold variation in Fe and Zn levels and up to 6.6-fold variation has been observed in beans and peas (Gregorio et al. 2000). Apparently, this genotypic variation is generally more reduced in tubers (White and Broadley 2009) and in fruits (Carvalho and Vasconcelos 2013; Hakala et al. 2003). Cultivated wheat germplasm has limited genetic variation in Se content, but this bottleneck has been overcome by crossing the cultivated wheat with wild wheat genotypes (Carvalho and Vasconcelos 2013; Lyons et al. 2005). Promisingly, seed banks and germplasm storage could play a major role in future nutrition-improvement approaches. The strengths and opportunities associated with the nutrition-improvement programs through conventional breeding have encouraged the commencement of many international programs to enrich the nutrient content of several crops, both to improve health and to prevent MNM.

3.3.2 Genomics-Assisted Breeding

Genomics-assisted breeding (GAB) is comprised of two types: marker-assisted selection (MAS, including marker-assisted backcrossing) and genomic selection (GS) (Varshney et al. 2014). MAS primarily involves the identification of molecular markers present within the nutrition-specific genes or quantitative trait loci (QTLs) and uses them to choose the cultivars which possess favorable alleles for enhanced nutrition quality. GS integrates the marker data and pedigree data of a training population to generate a prediction model to generate genomic estimated breeding values

(GEBVs). These GEBVs provide information about the potential of a particular cultivar as a parent in crossing and breeding. Thus, GAB enables the prediction of phenotypic performance of mature cultivars without performing extensive evaluation. Furthermore, GAB also permits maximum selection cycles and larger genetic gain per unit of time (Varshney et al. 2014).

Since the process of choosing crops for breeding has moved from phenotyping to genotyping-based methods, there has been a steady increase in the number of markers used for selection. The advancements in NGS has facilitated the genome-wide genotyping through development of large-scale markers. For example, foxtail millet (*Setaria italica*) was considered to be an orphan millet and a very limited number of markers had been developed for it (Gupta et al. 2011, 2012, 2013a). Later, the crop was designated as a *model* for genetic and genomic studies in bioenergy grasses (Lata et al. 2013) and the subsequent release of a draft genome sequence (Bennetzen et al. 2012; Zhang et al. 2012a) has resulted in large-scale development of different types of molecular markers such as microsatellites (Kumari et al. 2013; Pandey et al. 2013), intron-length polymorphic (Muthamilarasan et al. 2014b), miRNA-based (Yadav et al. 2014a) and transposable elements-based markers (Yadav et al. 2014b). In addition to the development of markers, their applications in GAB have also been demonstrated (Gupta et al. 2013b; Muthamilarasan and Prasad 2015) and these marker data were made available to the research community through open-access databases (Khan et al. 2014; Suresh et al. 2013).

Thus, NGS and high-throughput sequence analysis platforms have provided genome-wide marker coverage which enables researchers to evaluate the inheritance of an entire genome with nucleotide-level precision (Varshney et al. 2014). Taken together, GAB has the potential to leverage genotypic information which is relatively rapid, inexpensive and simple. Up to now, there has been no information available on utilizing GAB for improving nutrition content of crop plants; therefore, this should be immediately exploited.

3.3.3 Transgene-Based Approaches

Genetic engineering serves as a straightforward approach and a valid alternative for increasing the concentration and bioavailability of micronutrients in edible crop tissues when there is a lack of sufficient genotypic variation for the desired trait within the species or if the crop is not amenable to conventional breeding (Mayer et al. 2008). With the advent of NGS and high-throughput analysis platforms, the genomes of many staple crops have been sequenced (Muthamilarasan et al. 2013a) providing new opportunities for nutrition-enhancement programs. Redistributing micronutrients between tissues, enhancing the efficiency of biochemical pathways in edible tissue and reconstruction of selected pathways are the potential targets of transgenes. Instead of increasing the production or accumulation of micronutrients, some strategies have been established for the removal of *antinutrients* or inclusion of *promoter* substances to improve the bioavailability of micronutrients.

Transgene-based approaches to improve the mineral content of plants have mainly focused on Fe and Zn, the micronutrients most often lacking in human diet (Curie and Briat 2003; Palmgren et al. 2008). Overexpression of ferritin, an iron-storage protein had resulted in 3- to 4-fold increase of Fe levels in rice (Goto et al. 2000; Vasconcelos et al. 2003). Although the mineral levels are reduced during rice polishing, the Fe content and bioavailability of transgenic polished rice is still considerably higher in ferritin-enhanced lines (Murray-Kolb et al. 2002). Furthermore, there are many instances where transgene-based approach have led to generation of transgenic crops with increased concentration of vitamins or mineral elements (White and Broadley 2009). Golden Rice is the most popular genetically modified (GM) rice variety, wherein the carotenoid biosynthetic pathway has been reconstructed in non-carotenogenic endosperm tissue for producing β-carotene (provitamin A) in order to circumvent vitamin A deficiency (Paine et al. 2005). Promisingly, these GM rice varieties would supplement the recommended daily requirement of vitamin A (in the form of β-carotene) in 100–200 g of rice. This approach is also being successfully demonstrated in other crops such as maize, oranges, cauliflower, tomato, yellow potatoes and golden canola (White and Broadley 2009). Similarly, a GM carrot expressing high levels of a deregulated transporter which accumulated about twofold more Ca in the edible tissues was also developed (Carvalho and Vasconcelos 2013; Morris et al. 2008). In contrast with the Golden Rice, the feeding trials using this labeled carrot proved that Ca absorption was considerably increased in both animal models with diets consuming the GM carrot but not all the increased Ca was bioavailable (Murray-Kolb et al. 2002). This exemplifies the fact that an increase in nutrient content may not be directly translated into a similar increase in bioavailability (Carvalho and Vasconcelos 2013).

Although the transgene-based biofortification strategy has several common strengths and weaknesses with conventional plant breeding, this approach faces the threat of a low public acceptance and consequently a complex legal framework. In spite of these drawbacks, genetic engineering is now attempting *multigene transfer*, where several micronutrients can be added to the same plant (Carvalho and Vasconcelos 2013). Multivitamin maize is a very good example of this multigene transfer strategy, where GM maize was developed with high levels of β-carotene, ascorbate (vitamin C) and folate (vitamin B9) (Naqvi et al. 2009).

3.4 Progress in Enriching Cereals with Micronutrients

Significant progress has been made in developing nutrient-enriched cereals. The International Maize and Wheat Improvement Center (CIMMYT) along with the International Institute of Tropical Agriculture (IITA) and the National Agricultural Research and Extension Systems (South Africa) have initiated maize breeding programs for provitamin A. Germplasm screening exposed genetic variation for the target level (15 ppm) of provitamin A carotenoids in temperate maize, which was then bred into tropical varieties (Carvalho and Vasconcelos 2013). Recent progress

in marker-assisted selection has expedited the promptness and precision of finding the genes regulating traits of interest in maize. Notably, food processing and cooking methods result in provitamin A losses below 25 % (Li et al. 2010), whereas drying and dark storage at 25 °C for ~4 months may lead to a 25–60 % decay of provitamin A (Burt et al. 2010). Bioavailability (the conversion rate of β-carotene to retinol) was originally assumed to be 12 to 1, but nutrition studies have revealed more efficient bioconversion rates of 3 to 1 and 6.5 to 1 (Li et al. 2010; Muzhingi et al. 2011). Nutritional efficacy studies are underway worldwide and results are expected to provide clues to proceed with further research in this area.

In case of rice, the International Rice Research Institute (IRRI) and the Bangladesh Rice Research Institute (BRRI) have recently developed high-Zn rice varieties for Bangladesh and India (Saltzman et al. 2013). Notably, high-yielding rice varieties with more than 75 % of the target are undergoing field trials in Bangladesh and India and are expected to be released soon. Micronutrient-retention studies relative to Fe have determined that the Zn content of rice is not considerably reduced by parboiling and less so by milling, because Zn is distributed more homogenously throughout the brown rice grain (Liang et al. 2008). Controlled studies performed by BRRI quantified the loss of Zn from rice during milling and washing before cooking, which showed that ~10 % of the Zn in the milled grain was lost during washing prior to cooking (Juliano 1985). Another 10–14 % of the Zn may be lost during boiling of rice in an excessive volume of water, which is discarded prior to serving (Dipti 2012). To the present, there are no data on bioavailability and efficacy of the Zn-biofortified rice due to poor sensitivity of serum Zn concentration in response to relatively low amounts of additional Zn intake.

The Swiss Federal Institute of Technology was the first to develop Golden Rice and its research was advanced by Syngenta towards commercialization. Rice cultivars with higher levels of provitamin A, up to 37 ppm were produced and donated for use by the Golden Rice Network (Al-Babili and Beyer 2005). Currently, research on Golden Rice is being advanced by IRRI (Beyer 2010). Interestingly, bioavailability testing has confirmed that Golden Rice is an effective source of vitamin A for humans, with an estimated conversion rate of β-carotene to retinol of 3.8:1 (Tang et al. 2009). In addition, transgenic rice lines with high Fe content have been developed by the University of Melbourne and IRRI. Those contain 14 ppm Fe in the white rice grain and translocate Fe to accumulate in the endosperm (Johnson et al. 2011). Since the Fe is unlikely to be bound by phytic acid in the endosperm it would be bioavailable. Still, it has to pass numerous check-points and field trials before reaching the commercial market.

Wheat is a major source of dietary energy and protein for the global population. Its potential to contribute towards eliminating micronutrient-related malnutrition was recently realized and numerous efforts have been advanced towards biofortification of wheat for high Zn, Fe and Se (Velu et al. 2013). Targeted breeding of these biofortified varieties was initiated by exploiting available genetic diversity for Zn and Fe from wild relatives of cultivated wheat and synthetic hexaploid progenitors (Velu et al. 2013). The most promising and convincing results from the performance of competitive biofortified wheat lines demonstrated excellent adaptation in target

environments without compromising essential core agronomic traits. The Punjab Agricultural University, India is now assessing the Fe and Zn losses associated with traditional milling and cooking methods (ftp://ftp.fao.org/docrep/fao/005/y8346m/y8346m06.pdf). In addition, an independent study on absorption of Zn among Mexican women showed that total absorbed Zn was significantly higher from the biofortified variety of wheat as compared to non-biofortified wheat (Rosado et al. 2009). Additional absorption and efficacy research for Zn, Fe and Se are requisite to identify the bioavailability and validate the cultivars for genotype-specific variations.

Attempts to improve nutrition have also been initiated for several other crops. First, the International Center for Agricultural Research in the Dry Areas (ICARDA) is working towards biofortification of lentils for higher levels of Fe and Zn (http://www.icarda.org/south-asia-and-china-regional-program). In 2009, ICARDA began multilocation testing in Bangladesh, Ethiopia, India, Nepal and Syria. Notably, mineral-dense lentil varieties identified in early screening are already promoted for wide-scale cultivation in Bangladesh (http://www.icarda.org/south-asia-and-china-regional-program). Govind Ballabh Pant University of Agriculture and Technology, India is actively engaged in cowpea biofortification and has released two early-maturing high-Fe and Zn cowpea varieties, Pant Lobia-1 (2008) and Pant Lobia-2 (2010) (http://www.gbpuat.ac.in/research/DES%20Web%20Page.htm). These varieties are enrolled in the national seed multiplication system and seeds are available to farmers. The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) has developed Zn- and Fe-dense sorghum hybrids which are expected to undergo multilocational testing and on-farm adaptation trials in India (http://www.icrisat.org/crop-sorghum.htm). The International Potato Center (CIP) has developed high-Fe potato lines. Queensland University of Technology and the National Agricultural Research Organization of Uganda are working towards developing transgenic banana enriched with provitamin A and Fe (http://www.banana.go.ug/index.php/news/39-gm-bananas-could-cut-blindness-anaemia-in-east-africa). Bananas with up to 20 ppm provitamin A have been developed and trials are underway in Uganda (Namanya 2011).

3.5 Millets: A Store House of Nutrition

Similar to cereals, millets also belong to the grass family (Poaceae), but unlike cereals, millets possess certain nutritional and therapeutic properties, and are nutritionally superior to cereals (Muthamilarasan et al. 2015). Grains of millets are rich in micro and macronutrient contents and notably they have a low glycaemic index (GI) and high fiber content. Among millets, foxtail millet has higher protein and fiber contents of ~305 mg/g and 42.6 %, respectively. Proteins are the sole source of amino acids for humans whereas fibers enhance sugar and cholesterol metabolism to produce hypoglycemic and hypocholesterolaemic effects. Hence foxtail millet is highly beneficial for diabetics and for patients with cardiovascular diseases (Muthamilarasan et al. 2015). Notably, when compared

with major cereals such as rice and wheat, millets are rich in minerals (4.4 g/100 g in barnyard millet), calcium (344 mg/100 g in finger millet) and thiamine (590 mg/100 g in foxtail millet). Therefore, the available germplasm of all the millets should be screened for analyzing the physio-chemical properties of nutritional contents followed by integration of omics platform with genome-wide association analysis to identify high fidelity molecular markers such as SNPs and InDels. This also enables the identification of candidate genes controlling nutritional traits in millets.

The existence of synteny and conservation in the grass family could be exploited to perform a comparative study among millets and other cereals to trace the common genes involved in nutrition biosynthesis pathway. Although demonstrated recently in many crop plants, such studies are still lacking in millets and hence gaining insight into these aspects is highly essential not only for genetic improvement of millets for nutrition content but also of cereals. Once identified, these genes present in the diverse germplasm can be incorporated into the elite cultivars through either molecular breeding programs or transgene-based approaches to enhance the nutrition accumulation. The genetic close-relatedness of millets with major cereals such as rice, wheat, barley, sorghum and maize, could facilitate the introgression of these characteristics from millets into the major cereals and these nutritionally enhanced graminaceous crops would serve as a healthy diet for the global population.

3.6 Role of Omics in Improving Nutrition Contents

In the post-genomic era, deciphering the functional connections between genes, transcripts, proteins, metabolites and nutrients remains biology's greatest challenges. Recent technological advances in genomics, transcriptomics, proteomics and metabolomics will highly benefit the plant biofortification processes (Fig. 3.1). The integration of this knowledge will be more successful in defining appropriate biofortification strategies (Carvalho and Vasconcelos 2013). Genes controlling the tissue-specific nutrient accumulation and nutrient concentration have been identified using genomic tools, although the precise roles of these genes remain elusive. Certain genes may coordinate the uptake and transport of more than one mineral, and so the omic studies should be carefully applied in order to maintain mineral homeostasis.

As already mentioned, identification and characterization of the genes that control nutrient uptake, transport, storage and bioavailability is a tedious procedure. Recent advances in high-throughput sequencing/transcriptomics may expedite the study. For example, the high-throughput technologies such as pyrosequencing, microarray, serial analysis of gene expression (SAGE), suppression subtractive hybridization (SSH) and macroarray technology will provide a non-targeted, full spectrum analysis of all the genes expressed by a tissue at a given time point. Proteomic research has helped researchers understand the effects of proteins on plant mineral nutrient homeostasis. It seeks to observe the protein fluctuations under

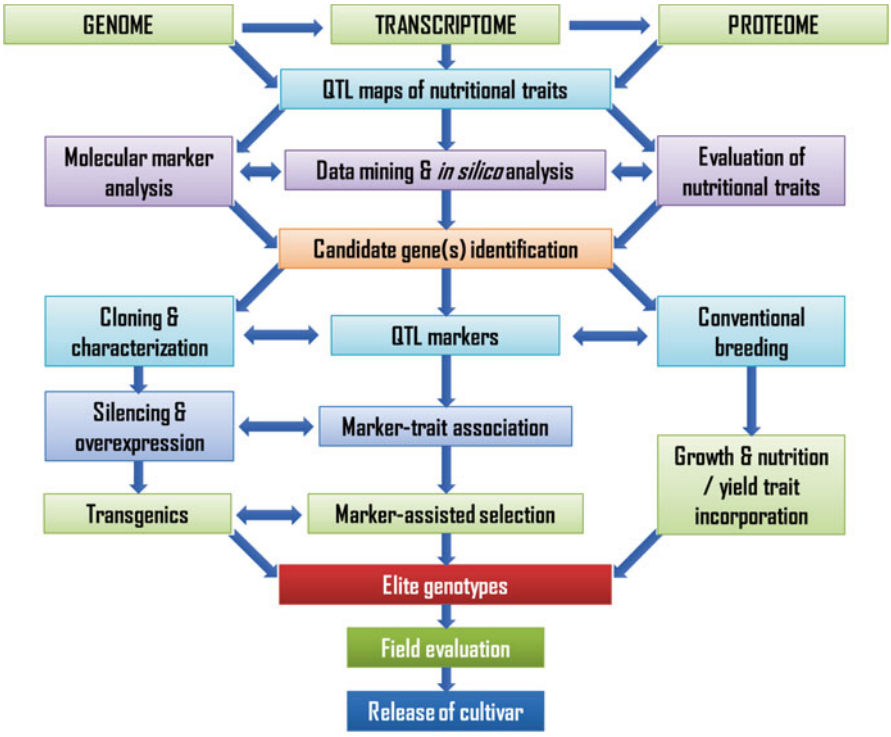

Fig. 3.1 Schematic diagram showing the integrated roles of omics technologies in crop improvement

variable developmental and environmental effects, as programmed by the genome, and mediated by the transcriptome (Carvalho and Vasconcelos 2013). Of note, a recent proteomics technology termed iTRAQ (isobaric tags for relative and absolute quantification) based on quantitative proteomics was used to analyze the microsomal proteins from *Arabidopsis* roots (Fukao et al. 2011). Other proteomic technologies used to study the protein chemistry include SDS-PAGE, mass spectrometry and protein chips.

In addition to genomics, transcriptomics and proteomics, metabolomics is also gaining momentum in biofortification studies. It provides a better understanding of the pathways responsible for the biosynthesis of nutritionally-relevant metabolites. Plants are reservoirs of chemically-diverse metabolites, which are usually present in a large range of concentrations (De Vos et al. 2007). Hence no single analytical method is able to extract and detect all the metabolites. Available techniques such as gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) and nuclear magnetic resonance (NMR) can be used for metabolite profiling.

Furthermore, it is worth mentioning that plants require at least 17 elements for proper growth and development out of the 92 naturally-occurring elements

identified on earth (Karley and White 2009). To study all these elements and identify the mechanisms that coordinately regulate them in response to genetic and environmental factors was performed using ionomics (Williams and Salt 2009). This was achieved using high-throughput inductively coupled plasma optical emission spectroscopy (ICP-OES) and inductively coupled plasma mass spectrometry (ICP-MS). In addition, the in silico protein analysis tools provided through ExPASy (http://www.expasy.org/), MIRA (Chevreux et al. 2004), Myrna (Langmead et al. 2010), MG-RAST (Mayer et al. 2008), Blast2Go (B2G) (Conesa et al. 2005), InterPro (Hunter et al. 2008), and Mascot (Perkins et al. 1999) will also expedite the proteomic research on nutritional aspects.

3.7 Conclusions and Prospects

In summary, it has been acknowledged that enhancing the nutritional levels of crop plants as such is a lengthy and multifaceted process, which involves a great deal of scientific and economical input. But the advances in omics technologies particularly genomics, have the potential to expedite this process. In this context, the present chapter exemplifies the different approaches of genomics-assisted nutrition enhancement by providing examples. A few success stories are reported in increasing the micronutrient-density and/or bioavailability in many plant-based foods through other methods have also been described. Taken together, it can be anticipated that many nutritional traits will be investigated through genomics and functional studies. Since the biological questions pertaining to nutritional traits cannot not be addressed in model systems such as *Arabidopsis*, experiments in native plants should be encouraged. This would clearly contribute to the long term goal of enhancing the nutrition levels of crop plants towards addressing global malnutrition.

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Chapter 4
Molecular Farming Using Transgenic Approaches

Ramandeep Kaur Jhinjer, Leela Verma, Shabir Hussain Wani, and Satbir Singh Gosal

Abstract Transgenic plants can be used for molecular farming for the production of recombinant pharmaceutical or industrial compounds. They offer attractive alternatives to produce low-cost recombinant pharmaceuticals or industrially-important proteins on a large scale. The feasibility of precise plant genetic manipulation, high-scale expression of recombinant proteins, rapid and easy scaling up, and convenient storage of raw material and less concern of contamination with human or animal pathogens during downstream processing has attracted biotechnologists to plastid and chloroplast engineering. Crop plants produce large amounts of biomass at low cost and require limited facilities. Molecular farming represents a novel source of molecular medicines, such as plasma proteins, enzymes, growth factors, vaccines and recombinant antibodies, whose medical applications are understood at the molecular level. Edible organs can be consumed as uncooked, unprocessed or partially-processed material, making them ideal for the production of recombinant subunit vaccines, nutraceuticals and antibodies designed for topical application. Based on this prominence, the present chapter is aimed to cover recent advancement for the production of plant pharmaceuticals.

Keywords Genetic engineering • Molecular farming • Pharmaceutical • Recombinant proteins • Vaccines

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4.1 Introduction

The use of plants for medicinal purposes is not new but genetic engineering of plants to produce desired biopharmaceuticals is quite recent. The growing of plants in agriculture to produce pharmaceutical or industrial compounds instead of food, feed or fiber is called *plant molecular farming*. The possibilities range from the manufacture of medical products, such as pharmaceuticals (drugs) and vaccines, to the production of products like biodegradable plastics (Kamenarova et al. 2005) and industrial chemicals (Paul et al. 2011; Sahu et al. 2014; Stoger et al. 2014). With the development of efficient genetic transformation methods, transgenic plants are now increasingly being used for the production of a variety of compounds such as monoclonal antibodies (Hiatt et al. 1989), blood substitutes (Magnuson et al. 1998), industrial proteins (Franken et al. 1997), parental therapeutics, pharmaceutical intermediates and antigens/antibodies (Walmsley and Arntzen 2000).

As the demand for biopharmaceuticals is increasing, they should be made available in significantly larger amounts. Currently, the cost of biopharmaceuticals limits their availability (Kayser and Warzecha 2012). Plant-derived biopharmaceuticals are cheap to produce and store. They are easy to scale up for mass production, and safer than those derived from animals (Ma and Wang 2012). Plant molecular farming is the use of genetically-modified plants to produce pharmaceutical products or industrial chemicals. These plants are developed by inserting new genes, usually from other species, so that a plant can produce the desired substance. Substances accumulate in specific parts of the plant, such as seeds or leaves and can be extracted and refined for use. Plants under investigation include potato, banana, wheat, tobacco, rice, soybean, spinach, maize, legumes, tomato and *Arabidopsis* (Jelaska et al. 2005). These plants could be used to fight diseases like cholera, measles, hepatitis-B, Norwalk virus, rabies virus and enterotoxigenic *Escherichia coli* (Thomas et al. 2002).

Transgenic plants that express foreign proteins of industrial or pharmaceutical value, represent an economical alternative to fermentation-based production systems (Valkova et al. 2013). The cost of vaccines is one of the factors preventing their wider use in vaccination, leaving thousands of children at risk of preventable diseases. First, the cost of purchasing and administering current vaccines is too high for many developing countries (Fischer et al. 2014). Second, compliance is limited by the inconvenience of needing trained personnel to administer injections and the reluctance of many individuals to receive injections. Third, a small but significant proportion of individuals vaccinated are not protected by current vaccines. The principal costs of most marketable vaccines are in production, packaging and delivery. Other related expenses are disposal of needles and syringes and refrigeration required during storage. These economic factors also prevent widespread vaccination of livestock, poultry and swine against avoidable diseases.

Plant vaccines are generally inexpensive to produce and thus can easily be made available in developing countries. For some vaccine antigens, transgenic plants may provide an ideal expression system in which transgenic plant material can be fed directly to people as an oral dose of recombinant vaccine. Edible plant vaccines are

effective as delivery vehicles for inducing oral immunization (Horn et al. 2004; Stoger et al. 2014). This is because production cost is low, effective safety, extraction and purification are not required. In addition, these vaccines are safe to store and transport, reducing the need for medical personnel and sterile injection conditions and dependence on foreign supply (Rathore and Shekhawat 2007). This chapter provides an overview of molecular farming prospects to produce various human health-promoting recombinant subunit vaccines, nutraceuticals and antibodies.

4.2 Transgenic Approaches

Genetic transformation technology has been proved to be a powerful tool for the production of plants with desired traits in many crops (Fig. 4.1). It promises to overcome some of the substantial agronomic and environmental problems that have not been solved using conventional plant breeding programs. Plant transformation mediated by *Agrobacterium tumefaciens*, plant pathogenic bacterium, has become the most commonly used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants. This soil bacterium possesses the natural ability to transform its host by delivering a well-defined DNA fragment, the transferred (T) DNA, of its tumor-inducing (Ti) plasmid into the host cell. Rapid progress in the area of crop biotechnology is mainly because of the development of efficient regeneration and suitable *Agrobacterium*-mediated transformation protocols for different crop species. Similar success could also be achieved in medicinal plants, which in turn could be used for the enhancement of

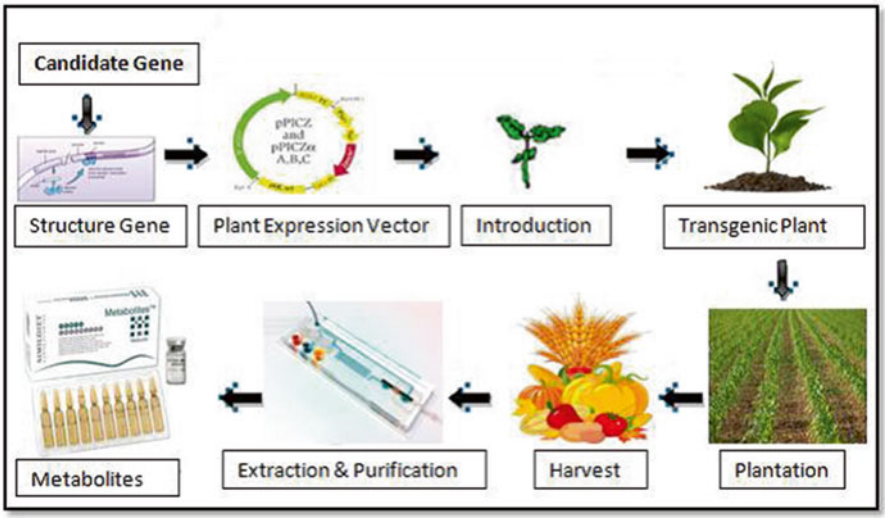

Fig. 4.1 Simplified representation of molecular farming (Sahu et al. 2014)

secondary metabolite content (Khan et al. 2009). *Agrobacterium*-mediated gene transfer has the advantage for allowing stable integration of defined DNA into the plant genome that generally results in lower copy number, fewer rearrangements and more stability of expression over generations than other transfection methods. Recent studies have shown that virus-based vectors can be efficiently used for high transient expression of foreign proteins in transfected plants and that non-Agrobacterium bacterial species can be used for the production of transgenic plants, laying the foundation for alternative tools for future plant biotechnology. Non-Agrobacterium species like *Rhizobium* sp. NGR234, *Sinorhizobium meliloti* and *Mesorhizobium loti*, are capable of genetically transforming different plant tissues and plant species (Broothaerts et al. 2005).

Another method of transformation, particle bombardment, was introduced in 1987, which involves the use of a modified shotgun to accelerate small diameter (1–4 μ) metal particles into plant cells at a velocity sufficient to penetrate the cell wall. There is no intrinsic limitation to the potential of particle bombardment since DNA is governed entirely by physical parameters. Different types of plant materials have been used as transformation targets including callus, cell suspension cultures and organized tissues such as immature embryos and meristems. The results indicate that biolistic transformation can lead to the transfer, expression and stable integration of a DNA fragment into chromosomal DNA. The biolistic method usually results in higher copy-number integration of the transgene than *Agrobacterium*, which can enhance expression. However, excessive copies or very high level expression of nuclear genes can cause gene silencing, resulting in low expression (Finnegan and McElroy 1994; Hobbs et al. 1993; Vaucheret et al. 1998). Thus it is important to select transgenic lines that carry only 1–3 copies of the transgene. The biolistic method is also used to introduce transgenes into the genome of the chloroplast (Svab et al. 1990). The high chloroplast genome copy number in plant cells contributes to enhanced recombinant protein expression. Some agronomically important plant species (e.g. soybean and most cereal grains) are recalcitrant to *Agrobacterium* transformation; for these plants, the biolistic method is frequently used (Sanford et al. 1993).

4.3 History of Molecular Farming

Molecular farming is as old as the first successfully transformed plant, because all of the genes have the potential of giving a protein product (Fischer et al. 2014). Initially human growth hormone and antibody chains were produced using transgenic tobacco and sunflower (Barta et al. 1986; Hiatt et al. 1989). The first marker genes have used in developing a transformation systems in plants is uids (Jefferson et al. 1987), which is now a molecular farming product (Kusnadi et al. 1998; Witcher et al. 1998). During (1988) reported the first human antibodies produced in plants. The first protein produced in plants for the specific purpose of extraction, purification, and sale was avidin. It is an egg protein with several important properties

(Hood et al. 1997). Zhong et al. (1999) detailed the production of aprotinin, one of the first molecularly-farmed pharmaceutical proteins produced in plants. Aprotinin can be used medically for wound closure and to suppress systemic inflammatory responses during surgery.

4.4 Molecular Farming Systems

Vaccine antigens can be produced in plants using two different systems: stable genetic transformation and transient expression. Stable transformation produces plants that can be propagated either vegetatively, or by seed resulting from sexual reproduction (Tacket et al. 1998, 2000). Transient expression uses a recombinant plant virus that carries the vaccine gene and by systemic infection, causes the plant to express the antigen (Koprowski and Yusibov 2001). The main advantage of transient expression with a plant-virus system is that virus replication amplifies gene copy number, resulting in a much higher level of expression than with stable transformation. However, the plant-virus systems may suffer from instability and loss of foreign genes larger than 1 kb. In addition, the need to individually inoculate each plant makes large-scale production laborious. Stable transformation causes integration of the recombinant DNA into the genome of either the nucleus or the chloroplast of the plant cell. Nuclear transformation is most often achieved with a plant pathogen such as *Agrobacterium tumefaciens* that can efficiently transport DNA into plant cells targeting the nucleus to cause chromosomal integration at random sites (Zambryski 1988). Stable transgene expression was recently described in the chloroplast-derived chromoplasts of tomato fruit (Ruf et al. 2001). A further advantage is that, as the chloroplast genome is inherited maternally in most plants, there is minimal danger of unintended spread of transgenes by pollen.

Currently there are four methods of protein production from plants: (1) nuclear transformation, (2) stable plastid transformation, (3) transient transformation and (4) stable transformation of a plant species grown hydroponically such that the transprotein is secreted into the medium and recovered.

The first and most common method of protein production from plants is nuclear transformation of a crop species. All the products available in the market today are based on this method (Fig. 4.1). The most-commonly used methods for transferring foreign genes into the plant cells, are *Agrobacterium tumefaciens* or particle bombardment, in which the genes are incorporated stably into the host nuclear genome. In grain crops, the protein product is normally accumulated in the seed (Delaney 2002). Seed can be stored without protein degradation even if unrefrigerated for a long time, and transportation can also be done at ambient temperature. The disadvantage of this type of transformation is higher manual labor requirements and lower yields.

The second method, stable plastid transformation of the genome, was first reported in a higher plant, tobacco, by Svab et al. (1990). The transgenic plastid genomes were products of a multistep process, involving DNA recombination, copy

correction and sorting out of plastid DNA copies. Tobacco is the only species in which plastid transformation has been established as routine (Daniell et al. 2002; Svab and Maliga 1993). Plastid genes are not usually transmitted through pollen so that outcrossing is not a major problem. But in this system protein stability will change over time even with refrigeration. Extraction and purification must be performed at very specific times following harvest. Large volume products and edible vaccines would not appear to be feasible using this system.

Transient transformation system, the third method, depends on the ability of recombinant plant viruses such as tobacco mosaic virus (TMV) to infect tobacco plants and then transiently express a target protein in the plant tissue. The protein will accumulate in the interstitial spaces and the interstitial fluid can then be collected by centrifugation under vacuum. TMV can be easily manipulated genetically and the infection process is rapid. Small quantities of the target protein can be obtained within several weeks. But this type of transformation is not suitable for a protein needed in large quantities. The product must be processed immediately as storage will cause degradation of the plant tissue (Stoger et al. 2014).

The fourth method of protein production from plants uses hydroponic systems (floating systems), with transgenic plants containing a gene coding for production of the target protein. It is intermediate between suspension and whole plant cultures, based on the use of whole organisms (micro algae, moss or aquatic plants) that are fully or partly in contact with a culture medium and also have the advantage of being fully contained and allowing the secretion-based recovery of the product (Decker et al. 2014; Mathieu-Rivet et al. 2014; Raskin 2000). Purification of the desired product is considerably easier since no tissue disruption is needed and the quantity of contaminating proteins is low. But this system is also unsuitable to producing large quantities of any protein product. In addition, hydroponic facilities are relatively expensive to operate.

4.5 Classes of Proteins Within Molecular Farming

Biotechnology involves the efficient utilization of biological material for rich harvest. Plant biotechnology, especially the genetic engineering of plants, has been developed to such an extent that cloned gene(s) synthesized in the laboratory can be introduced into plants which stably express the genes in the resulting transgenic plants and are also transmitted to the next generations. The transgenic approach has resulted in the release of several superior varieties of different crops for commercial production in many countries of the world. Plants have traditionally been a major source of medicines; for example, the Ayurvedic system depends upon plant-based medicines. With the advent of innovative approaches, plants have been given renewed possibilities to produce preventive drugs. Proteins currently being produced in plants for molecular farming purposes can be categorized into four broad areas: (1) parental therapeutics and pharmaceutical intermediates, (2) monoclonal antibodies (MAbs), (3) industrial proteins (e.g. enzymes) and (4) antigens for edible vaccines.

4.5.1 Parental Therapeutics and Pharmaceutical Intermediates

This group includes all proteins used directly as pharmaceuticals. Such proteins include products like thrombin and collagen (therapeutics), and trypsin and aprotinin (intermediates). Many proteins in this category have been expressed in tobacco. However, there are some notable exceptions; for instance, rice has been used for the production of human α-interferon (Zhu et al. 1994), canola for hirudin (Parmenter et al. 1995) and α-1 antitrypsin (Terashima et al. 1999) and maize for bovine aprotinin (Azzoni et al. 2002; Zhong et al. 1999). Recombinant human glycoproteins synthesized in plants show much greater similarity to their native counterparts in terms of N-glycan structure compared to the same proteins produced in yeast, bacteria or filamentous fungi (Obembe et al. 2011). Recent reports indicate that rice is a suitable host for recombinant protein due to its competitive yield of certain recombinant proteins, such as human serum albumin, cytokines compared to other plant expression systems such as tobacco cell cultures and potato tubers (Kuo et al. 2013). One of the first publications involving producing HAS (human serum albumin) using a rice suspension cell system with an inducible promoter αAmy3/RAmy3D, reported a maximum yield of 76.4 mg/l of medium after 4 days of sucrose starvation (Huang et al. 2005). There is more than a sixfold yield advantage compared to HSA produced using tobacco suspension cell, which was 11.88 mg/l (Sun et al. 2011). Alternatively, a high yield of recombinant HSA has also been produced in rice endosperm. An endosperm-specific promoter, Gt13a, and its signal peptide were used to direct expressed protein into protein storage vacuoles, and a maximum yield of 2.75 g/kg of brown rice was obtained (He et al. 2011). This is a significant yield improvement over production of HSA using potato tubers (0.01 g/kg of fresh weight) and well above the threshold for cost-effective industrial production, which was 0.1 g/kg of fresh weight (Farran et al. 2002).

The structural study of HSA shows that recombinant HSA (OsrHSA) produced using a rice expression is structurally equivalent to plasma HSA (He et al. 2011). The first report of human GM-CSF (hGM-CSF) production in rice was in 2003 through suspension cell cultures, and the maximum yield obtained was 129 mg/l (Shin et al. 2003). Since then, improvements have been made in rice suspension cell systems producing hGM-CSF by using methods such as humanizing *N*-glycan structure and increasing yield by two to threefold through reduction of endogenous α-amylase expression, co-expression of proteinase inhibitor and suppression of cellular cysteine proteinase (Kim et al. 2008a, b, c, d; Shin et al. 2011). The protein is also produced in other expression systems such as rice seeds and tobacco seeds using rice glutelin promoters Gt1 and Gt3. The maximum yields were 1.3 % of total soluble protein in rice seeds and 0.03 % of total soluble protein in tobacco seeds (Sardana et al. 2002, 2007). Mouse GM-CSF (mGM-CSF) was successfully produced using rice suspension cells, with yield of 24.6 mg/l of medium (Liu et al. 2012). The same glycoprotein was also produced in tobacco leaves, and the yield was 19 μg/g of fresh leaves (Gora-Sochacka et al. 2010). Interleukin-10 (IL-10) has been successfully produced in rice seeds using Glub-1 promoter and its signal

peptide to localize the protein inside seeds exclusively. The product is determined to be un-glycosylated and the yield of final purified protein was 2 mg per 40 g of rice used (50 μg/g). The biological activity of recombinant IL-10 was confirmed using mouse bone marrow dendritic cells (Fujiwara et al. 2010). The protein was also produced inside tobacco leaves, and the yield was 37.0 μg/g of fresh leaves (Bortesi et al. 2009). Production of class II interferon INF-γ has been performed in rice suspension cells using both constitutive maize ubiquitin promoter and inducible rice αAmy3/RAmy3D promoter, and the biological activity has been confirmed using human A549 cell line against dengue virus. A α-amylase signal peptide was added to both in order to allow for secretion of recombinant INF-γ into the culture medium. The highest yield obtained from the culturing medium of the ubiquitin promoter driven system was 12 and 17.4 ng/mL in αAmy3/RAmy3D promoter driven system, and yield found inside the cell was 699.79 ng/g of fresh cell weight for the ubiquitin promoter driven system and 131.6 ng/g of fresh cell weight for the αAmy3/RAmy3D promoter driven system (Chen et al. 2004).

Production of human α1-antitrypsin (hAAT) in recombinant rice suspension cells was first achieved in 2000, with a yield of 120 mg/l, cultured in 30 mL of medium with 10 % (v/v) of cell density (Huang et al. 2001). Recombinant hGH (human growth hormone) has been successfully produced in rice suspension cells using αAmy3/RAmy3D as a promoter. The maximum yield is 57 mg/l in a culture medium, and the biological activity of the recombinant protein has been confirmed using Nb2 node lymphoma cells, whose proliferation and growth depend on the presence of hGH (Kim et al. 2008f). A fungal immunomodulatory protein (FIP-fve) was isolated from golden needle mushroom (*Flarnmulina velutipes*) and reported to inhibit allergy reactions in mice and regulate Th2 cytokines (Hsieh et al. 2003). Recombinant FIP-fve fused with the antigen Der p 2 (OsDp2Fve) has been produced using rice suspension cells under a control of α-amylase (αAmy8) promoter, and the yield was about 7.5 μg/mL (10.5 % of total protein) (Su et al. 2012).

4.5.2 Monoclonal Antibodies

Vaccines for active as well as passive immunization have been produced in plants. Plantibodies (plant antibodies) are the human/animal antibodies made by and in transgenic plants. Using antibodies as drugs is not new, but manufacturing them in plants is. So far most have failed, for two reasons. First, many early antibody drugs either did not work or provoked severe allergic reactions. The new drug may be effective, but it will not be cheap; cost is the second barrier these medicines face. Maize is not the only crop that can mimic human cells. Agracetus (Monsanto, USA) is also cultivating soybeans that contain human antibodies against herpes simplex virus 2, a culprit in venereal disease. The concept of passive immunization (application of antibodies) is been explored by producing complete antibodies in the plants. In order to achieve this, independent transgenic plants producing a single chain are crossed to obtain hybrid plant producing complete antibodies consisting of light and

heavy chains (Fig. 4.2), (Wycoff 2004). Alternatively, antibody producing plants could directly be generated through cotransformation with genes responsible for light and heavy chain sub units. Transformed plant cells have been found to produce and assemble the antibodies, correctly. An antibody yield of up to 5 % of the total plant protein has been observed in transgenic plants. Agricultural plant production offers the most cost effective and large scale protein production system. It has been estimated that by expressing an antibody in soybean even at the level of 1 % of total protein, 1 kg of antibody could be produced for approximately USD 100.

This group includes all antibody forms [immunoglobin G (IgG); immunoglobin A (IgA); immunoglobin M (IgM); secretory IgA; etc.) and antibody fragments (Fv). They can be produced in plants in both glycosylated and non-glycosylated forms. These plant-derived MAbs (plantibodies) have the potential of alleviating the serious production bottleneck that currently exists as dozens of new MAb products attempt to reach the marketplace. Examples of plant-derived MAbs in product development include a-caries for prevention of dental decay and a-herpes for prevention of herpes transmission. Guy's Hospital London and the Salk Institute (USA) have produced mucosal antibodies against a tooth-decaying bacterium *Streptococcus mutans*. Tobacco-derived antibodies when applied to mucosal surface, prevented infection. Attempts are now being made to produce these antibodies at a large scale with an aim to incorporate them into toothpaste to battle streptococcal decay.

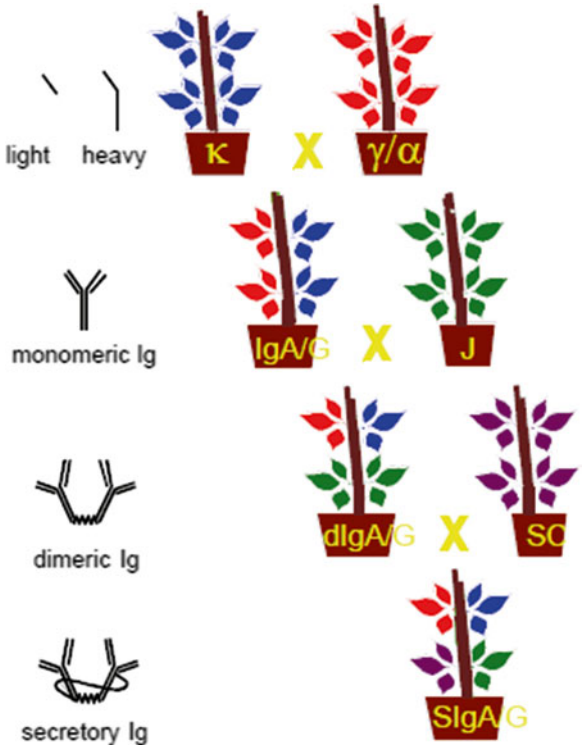

Fig. 4.2 Schematic showing the sequential crossing of individual plants expressing kappa chain, the chimeric IgA/G heavy chain, J chain and secretory component to produce IgA/G, dimeric IgA/G and secretory IgA/G (Wycoff 2004)

Aracetus (USA) has developed a transgenic soybean that produces a tumor reactive monoclonal antibody called BR96 which can be tried as a drug carrier to treat breast, colon, ovarian and lung cancers. Transgenic plants have been used for the production of antibodies directed against dental caries, rheumatoid arthritis, cholera, *Escherichia coli* diarrhea, malaria, certain cancers, Norwalk virus, HIV, rhinovirus, influenza, hepatitis B virus and herpes simplex virus (Thomas et al. 2002).

Functional antibodies produced in tobacco plants were first reported over a decade ago (1989). The basic protocol used to generate these plantibodies involved the independent cloning of H and L chains of antibody genes in *Agrobacterium tumefaciens* vectors followed by transformation of plant tissue in vitro with the recombinant bacterium. The first recombinant proteins produced in plants were progeny of the cross of two individual transgenic plants, tobacco and sunflower, expressing single immunoglobulin gamma and kappa chains (Hiatt et al. 1989). The production of antibodies has been reported by a number of academic laboratories and the subject has been reviewed extensively (Hood et al. 2002; Larrick et al. 2001; Schilberg et al. 2002; Stoger et al. 2002a, b). The progressive improvement of expression vectors for plantibodies, purification strategies, and increase in transformable crop species, lead to almost limitless availability of inexpensive recombinant immunoglobulins free of human pathogens for human and animal therapy.

Klaus described the production of MAbs (During 1988). This work demonstrated expression of the B 1–8 light and heavy chains in transgenic tobacco and then showed assembly of the full MAb. Unexpectedly, a high percentage of MAb of hybrid mouse line (B 1–8) was found in the chloroplasts. Hiatt et al. (1989), then at the Scripps Research Institute (La Jolla, Calif.), crossed transgenic tobacco containing gamma or kappa immunoglobulin chains and obtained progeny having fully functional MAb. Cabanes-Macheteau et al. (1999) reported the specific glycosylation composition of a mouse IgG expressed in tobacco plants. Frigerio et al. (2000) described the vacuolar accumulation of a secretory IgA in tobacco. Verch et al. (1998) used a plant virus vector to infect tobacco and produce a full-length MAb directed towards colon cancer antigen. One of the first plant-derived MAb products expected to reach the market is one directed against dental caries. Designated CaroRx, this secretory IgA (sIgA) inhibits the binding of the oral pathogen *Streptococcus mutans* to teeth. As reviewed by Larrick et al. (2001), the plant derived MAb was extremely effective in reducing colonization by *S. mutans* using passive immunization, and even prevented re-colonization for up to 2 years. Another plant-derived MAb product expected to reach the market is one directed against genital herpes. Zeitlin et al. (1998) reported the production of anti-herpes humanized MAb in soybean and compared this MAb with the same MAb produced in mammalian cell culture in a mouse model. The two MAbs protected the mice against herpes simplex virus-2 equally well, proving again that differences in glycosylation of transproteins do not significantly reduce efficacy in many cases. This potential product is now being produced in maize. Recombinant human cytotoxic T-lymphocyte antigen 4-immunoglobulin (hCTLA4Ig) has been successfully produced in rice suspension cells using αAmy3/RAmy3D promoter with maximum yield of 31.4 mg/l in liquid medium (Lee et al. 2007). To optimize sIgA (secretory

immunoglobulin A) production in plants, Juarez et al. (2013) tested the combinatorial expression of 16 versions of a human sIgA against the VP8* rotavirus antigen in *Nicotiana benthamiana*, using the GoldenBraid multigene assembly system. From the analysis of the anti-VP8* activity, it was concluded that those sIgA versions carrying HCα1 and LCλ provided the highest yields. Another antibody, single-chain Fv antibody (ScFvT84.66) under the control of maize Ubiquitin-1 promoter, has also been expressed in the leaves and calli of transgenic rice, and the yields were 29 μg/g and 3.8 μg/g of fresh weight of leaves and calli, respectively (Stoger et al. 2000; Torres et al. 1999).

4.5.3 Industrial Proteins-Enzymes

Expression of industrial enzymes in transgenic plants offers an alternative system to fungal fermentation for large-scale production. Very high levels of expression are required to make the enzymes cost-effective. Transgenic maize plants were generated using an *Agrobacterium*-mediated system. This group includes hydrolases, encompassing both glycosidases and proteases (Fischer et al. 2014). Oxidoreductase enzymes such as laccase, a fungal enzyme used in fiber bleaching and bioglue of wood products (Bailey et al. 2004; Hood et al. 2003), represent a separate class of industrial enzymes. Enzymes involved in biomass conversion for the purposes of producing ethanol are candidates for molecular farming. Recombinant egg white avidin and bacterial B-glucuronidase (GUS) from transgenic maize have been commercially produced. High levels of expression were obtained in seed by employing the ubiquitin promoter from maize. The recombinant proteins exhibited activities that were indistinguishable from their native counterparts, (Ma et al. 2003). All of these products are used in very large quantities and must therefore be produced very inexpensively (Hood et al. 1999). A major hindrance to the molecular farming of these products is the large land area needed. One of the most important driving factors has been yield improvement, as product yield has a significant impact on economic feasibility. Strategies to improve the recombinant protein yield in plants include the development of novel promoters, the improvement of protein stability and accumulation through the use of signals that target the protein to intracellular compartments (Schillberg et al. 2013), and the improvement of downstream processing technologies (Menkhaus et al. 2004).

4.5.4 Antigens for Edible Vaccines

Vaccines have played an appreciable role in controlling several human and animal diseases. Some major human diseases like diphtheria, tetanus, yellow fever, measles, mumps, rubella and polio have been largely controlled by vaccination (Hugh et al. 2002). However, according to the World Health Organization (2013), 16 % of

children worldwide are still not fully immunized against diphtheria, pertussis (whooping cough), tetanus, polio, measles and tuberculosis (TB). Moreover, the classic vaccines pose a risk that micro-organism may somehow spring back to life, causing disease again. For that reason vaccine today is produced as a subunit preparation, composed primarily of antigenic protein separated from pathogen genes. On their own the protein are not infectious. Sub-unit vaccines are however very expensive, because they are produced in cultures of bacterial or animal cells and have to be purified before use and also need refrigeration during storage and transport. Expression of antigens as vaccine in transgenic plants is a convenient and inexpensive source for these immunotherapeutic molecules (Kamenarova et al. 2005). Charles Arntzen (the first to use the phrase *edible vaccine*), with Hugh Mason and colleagues have pioneered the field with work on hepatitis B and heat labile toxin, B subunit, in tobacco plants and potato tubers (Arntzen et al. 2005).

Active immunization means the expression of antigens in plant parts which can preferably be consumed in a raw form, to avoid denaturation of proteins during cooking. The antigens thus expressed in edible plant parts such as potato tubers and banana fruit can stimulate immune response against a particular pathogen attack and prevent the disease/disorder in humans/animals (Sahu et al. 2014). This idea of edible vaccines was conceived by Charles J. Arntzen of USA in the early 1990s. Since then several antigens have been expressed in plants. Hepatitis B virus surface antigen has been expressed in tobacco, potato and lettuce. Hepatitis B kills more than one million people each year and is a leading cause of liver cancer. Likewise, edible vaccines have been produced against viral and bacterial diarrhea. Viral diarrhea is caused by Norwalk virus (NV), a calicivirus that causes epidemic acute gastroenteritis, a leading cause of food-borne illness in US. The bacterial diarrhea is caused by enterotoxigenic *Escherichia coli* (ETEC), which upon infection secretes toxins in the gut, which include heat labile exterotoxin (LT). The antigens for Norwalk virus capsid protein and *E. coli* LT B subunit (LT-B) have been expressed in potato and tobacco. Likewise, antigens for cholera toxin B subunit (CT-B), rabies virus glycoproteins (G-protein) and respiratory syncytial virus have been expressed in potato, tobacco and tomato, respectively. The edible vaccines have also been developed for livestock. The antigens for VPI protein of foot and mouth disease have been expressed in *Arabidopsis*. Other vaccines in the pipe line include vaccine for transmissible gastroenteritis virus (TGEV) in swine, which is a major cause of sickness and death in young pigs, antigens against mink enteric virus which do not affect humans. Extensive studies on development of edible vaccine against human papilloma virus type 16 (HPV 16) which causes cervical cancer in women has also been done. Until now, no conventional vaccine is available for HPV, so the outcome will be quite rewarding. Moreover, the chickens are being genetically engineered to lay eggs containing anti-cancer drugs. Likewise, in preliminary studies conducted in Bangalore, the rabies vaccine has been expressed in muskmelon. According to World Health Organization, rabies claims 70,000 lives every year and most of the victims are from developing countries. The development of edible vaccine could be a major breakthrough (Hugh et al. 2002).

The main goal of an oral vaccine is the induction of a mucosal immune response (Das 2009) and a subsequent systemic immune response. As a pre-requisite for testing these vaccines in humans, pre-clinical studies have been conducted in mice. Orally immunized mice with transgenic extracts have exhibited a strong mucosal and systemic immune response. Many pathogens enter the body through the nose, mouth and other openings. Hence the first defenses they encounter are those in mucous membranes that line the airways, the digestive tract and reproductive tract. When mucosal immune response is effective, it generates molecules known as secretary antibodies that neutralize any pathogen that it finds in its way. In 1998, the National Institute of Allergin and Infectious diseases (NIAID) demonstrated that an edible vaccine could stimulate an immune response in humans as well, which was a major milestone in active immunization. The volunteers, who ate genetically engineered potatoes containing toxin secreted by *Escherichia coli* bacterium c.o. of diarrhea, showed fourfold rise in serum and intestinal antibodies. Food vaccines might also help to suppress auto-immunity in which the body's defenses mistakenly attack normal, uninfected tissues. Among autoimmune disorders that might be prevented are type I diabetes, multiple sclerosis and rheumatoid arthritis. The type I diabetes is severe among children. It results from autoimmune destruction of the insulin producing beta cells, of the pancreas. The loss of beta cells eventually leads to drastic shortage of insulin, a hormone needed to help cells take up sugar from the blood for energy. The loss results in high blood sugar levels. Plant-based vaccines have been developed in potatoes, containing insulin or glutamic acid decarboxylase (GAD). Feeding of vaccines to mice helped to delay the onset of high blood sugar.

Compared to traditional commercial vaccines, edible vaccines have several advantages. They are relatively easy to produce at a low cost, and without the need of fermentation and protein purification steps, which are mandatory for conventional vaccine production. Unlike mammalian-derived rDNA drugs, plant-derived antibodies, vaccines and other proteins are particularly advantageous since they are free of mammalian viral vectors and human pathogens (Schillberg et al. 2013). Edible vaccines are easy to administer, as the plant products e.g. fruits/vegetables are eaten raw and there is no need of painful intramuscular/intravenous injections. Moreover, they are safe compared to conventional vaccines, as there is no risk of infection/contamination from the use of non-sterile needles (Lamphear et al. 2004). Advantageously, the edible vaccines do not need refrigerated storage, so they can be easily and cheaply stored, transported and administered to poor populations in real need of vaccination. The plants proposed thus far for edible vaccine production, like potato and banana, can be grown widely. Thus the development of edible vaccines is timely and useful development.

However, there are some questions which still need to be answered before commercial release of these vaccines. These include standardization of appropriate dosage to be consumed, environmental impact, need for high levels of expression of targeted proteins, ways to overcome possible degradation of expressed proteins during storage of fruits/vegetables, appropriate source of food to be used as edible vaccine and also the appropriate time and tissue of expression of the proteins. Extensive

studies are underway to answer all these questions favorably. Moreover, the avenues for commercialization of edible vaccines are being opened worldwide, as evident from the issuing of a US patent to a private firm, Prodigene, for the use of plants to develop oral vaccines that can immunize humans and animals against viral diseases. Thus the production of edible vaccines can be conceived as an attractive and an additional tool for vaccine production, which can relieve humankind of several diseases and disorders.

4.5.5 Production of Other Proteins of Medical Relevance

Proteins of medical relevance include the milk proteins ß-casein, lactoferrin and lysozyme, which can be used to improve child health, and protein polymers that could be used in surgery and tissue replacement (Ma et al. 2003). Expression of thioredoxin in foods such as cereal grains would increase the digestibility of proteins and thereby reduce their allergenicity (Thomas et al. 2002). It has been shown that human collagen can be produced in transgenic tobacco plants and that the protein is spontaneously processed and assembled into its typical triple-helical conformation. Due to a shortage of plasma and low expression levels of recombinant rbFGF (fibroblast growth factor) in conventional gene expression systems, An et al. (2013) explored the production of recombinant rbFGF in rice grains (*Oryza sativa* bFGF, OsrbFGF). An expression level of up to 185.66 mg/kg in brown rice was obtained. The functional assay of OsrbFGF indicated that the stimulating cell proliferation activity on NIH/3T3 was the same as with commercialized rbFGF. Wound healing in vivo of OsrbFGF is equivalent to commercialized rbFGF.

4.6 Host Plant Selection for Edible Vaccines

Potatoes have been frequently used as a convenient model system because transgenic lines can be efficiently produced using *Agrobacterium*-mediated DNA delivery. However, a more palatable system is preferred, as the vaccine must be consumed raw to prevent heat denaturation of the antigens. The tomato is an attractive alternative. Several plant species (dicot, monocot, food and non-food, leafy crops, cereals and legumes) have been used for the expression of recombinant proteins (Table 4.1). Banana is attractive because it is widely grown in developing countries and an efficient transformation system has been reported (May et al. 1995). From an economic point, soybean, alfalfa and maize are among the most efficient plant systems for recombinant protein production. Kusnadi et al. (1997) reported the production, purification, and characterization of recombinant *Escherichia coli* GUS and chicken egg-white avidin from transgenic maize seed.

Table 4.1 Different plant host systems in plant molecular farming

Plant	Preferred site of expression	Advantages	Disadvantages	Vaccine against	References
Medicago sativa	Leaf	Relatively efficient transformation system	Potential for outcrossing in field	Bacterial pathogen *Mannheimia haemolytica*	Khoudi et al. (1999)
		High protein content in leaves			
		Leaves edible uncooked			
Musa spp	Fruit	Excellent digestibility	Trees take 2–3 years to mature	Hepatitis B virus	Sunil Kumar et al. (2005)
		Palatability and availability throughout the year	Fruit spoils rapidly after ripening		
		Do not need cooking	Contains very little protein		
		Proteins not destroyed even if cooked inexpensive	Very expensive in greenhouse		
		Grown widely in developing countries			
Legumes and cereals (*Zea mays*, *Oryza sativa*, *Triticum aestivum*, *Glycine max*, *Hordeum vulgare*)	Seeds	Production technology widely established	Inefficient transformation systems	Japanese cedar pollen peptide in rice. Enterotoxigenic *Escherichia coli* fimbrial subunit in soybean. Heat-labile enterotoxin B subunit in maize	Zhong et al. (1999), Huang et al. (2005), Schillberg et al. (2005), and Ding et al. (2006)
		High protein content in seeds	Heating or cooking for human use might cause denaturation and poor immunogenicity of vaccine		
		Stable protein in stored seeds	Potential for outcrossing in field for some species		
		Well-suited for animal vaccines			
		Industrial seed processing well established			

(continued)

Table 4.1 (continued)

Plant	Preferred site of expression	Advantages	Disadvantages	Vaccine against	References
Solanum tuberosum	Tuber	Efficient transformation system	Raw potatoes are not palatable and cooking destroys protein antigens	Vaccine for cholera	Mason et al. (1996)
		Dominated clinical trials	Relatively low tuber protein content		
		Tuber-specific promoters available			
		Stored for long periods without refrigeration			
Nicotiana sp.	Leaf	Easy purification of antibodies stored in the seeds	Produces toxic compounds	Hepatitis B virus	Mason et al. (1996)
		Efficient transformation system available			
		Low cost preserving system			
S. lycopersicum	Fruit	Relatively efficient transformation system	Fruits spoils readily	Norwalk virus	Mor et al. (2001)
		Fruit is edible raw	Relatively low fruit protein content	HIV virus	
		Cultivated broadly			
		Overcome the spoilage problem by freeze-drying technology			

4.6.1 Vaccine Production in Fruit Crops

Several studies have reported the production of transgenic tomato plants for the expression of viral antigens, including rabies virus, foot-and-mouth disease virus, human papilloma virus, *Yersinia pestis* and for the production of therapeutic proteins. Many investigations have focused on the development of novel mucosal vaccines against HIV and HBV (Hepatitis B virus) in transgenic tomato plants. These tomato-made vaccines are proposed to be inexpensive, heat stable and easy to administer (Rigano et al. 2013). For example, a fruit-based edible subunit vaccine against RSV was developed by expressing the RSV fusion (F) protein gene in transgenic tomato plants. The F-gene was expressed in ripening tomato fruit. Oral immunization of mice with ripe transgenic tomato fruits led to the induction of both serum and mucosal RSV-F specific antibodies. Serum antibodies showed an increased titer when the immunized mice were exposed to inactivated RSV antigen (Sandhu et al. 2000). Shchelkunov et al. (2006) expressed the synthetic chimeric gene TBI-HBS encoding immunogenic ENV and GAG HIV-1 epitopes and the surface protein antigen (HBsAg) of hepatitis B virus in transgenic tomato plants and demonstrated that in mice oral administration of dried tomato tissues stimulated both serum and secretory HIV- and HBV-specific antibodies. Ramirez et al. (2007) expressed the HIV-1 Tat protein in transgenic tomato fruits and demonstrated that the orally delivered tomato-based vaccine raised mucosal IgAs and induced serum IgGs with neutralizing activity in mice.

Cueno et al. (2010) demonstrated the preferential expression of a Tat-GUS fusion protein produced in tomato plants. Protein extracts intradermally injected into BALB/c mice were found to induce both humoral and cellular immune responses. In addition, Zhou et al. (2008) expressed the HIV antigens p24 and Nef from the plastid genome of tomato. HIV antigen accumulation reached values of approximately 40 % of total leaf protein and 2.5 % of total protein in green tomatoes. Bacterial antigens have also been expressed in transgenic tomato plants. One example is the expression of a plant-optimized synthetic gene encoding the multiepitopic protein sDTP (diphtheria-pertussis-tetanus). The synthetic gene contained six DTP immunoprotective exotoxin epitopes and two adjuvants (Soria-Guerra et al. 2007). After that same group demonstrated that in mice, three oral doses with freeze-dried material from the tomato-derived multicomponent vaccine elicited specific systemic and mucosal antibody responses (Soria-Guerra et al. 2011). Tomato-made vaccines have also been used as a novel strategy for the development of vaccines against cholera. Oral vaccines would be particularly suitable to protect against pathogens that infect through intestinal surfaces since this delivery route best induces a mucosal immune response (Rigano et al. 2003; Salyaev et al. 2010). Some other examples are the production in transgenic tomato plants of: (1) CTB (Cholera toxin B subunit), (2) TCPA (toxin co-regulated pilus subunit A) of *Vibrio cholera* and its immunogenic epitopes P4 or P6 fused to CTB and (3) ACFA (accessory colonization factor subunit A) of *Vibrio cholerae* and ACFA fused to CTB (Rigano et al. 2013). Jha et al. (2012) demonstrated the feasibility of using tomato plants for the production of stable, glycosylated and biologically active recombinant human α1-proteinase inhibitor.

In addition, Kim et al. (2012) reported the stable production of human β-secretase in transgenic ripe tomato fruits. The capacity of fleshy fruits to accumulate functional antibody has been demonstrated by Juarez et al. (2012). The authors described the production of transgenic tomato plants expressing a recombinant human immunoglobulin A selected against the VP8* peptide of the model rotavirus strain SA11. Minimally processed fruit-derived products showed anti-VP8* binding activity and strongly inhibited virus infection in an in vitro virus neutralization assay. In addition, their paper dealt with the concerns often raised regarding possible contamination of the food chain with transgenic materials. In order to label the transgenic lines expressing the antibodies, sexual crossing were made with a transgenic tomato line expressing the genes encoding snapdragon (*Anthirrhinum majus*) Rosea1 and Delila transcription factors. These transcription factors ectopically activate anthocyanin biosynthesis in tomato fruit. The resulting purple-colored extracts from the transgenic fruit contained high levels of recombinant antirotavirus neutralizing human IgA in combination with increased amounts of health-promoting anthocyanins.

4.6.2 Vaccine Production in Tuber Crops

Transgenic potato tubers as bioreactors offer advantages such as long-term storage tissue, abundant biomass, short growth cycle, high nutritional value and the high stability of recombinant proteins accumulated in the tubers during a long period of storage. Potato has been used in recent years as a model system for the expression of bacterial, viral antigens and autoantigens and preliminary results from human clinical trial studies conducted with potato-based vaccines were promising. In addition, mucosal immunity has been induced by orally administered transgenic potato plants (Rigano et al. 2013). Warzecha et al. (2003) demonstrated that ingestion of transgenic tubers expressing the HPV11 L1 capsid protein activated anti-VLP immune responses that can be boosted by subsequent administration of purified insect cell-derived VLPs. Chen and Liu (2011) produced transgenic potato plants expressing GP5 protein of the porcine reproductive and respiratory syndrome virus (PRRSV) and showed that, in mice, oral administration of crude protein extracts from transgenic tubers elicited both serum and gut mucosal-specific antibodies.

Potato plants have also been used to produce several therapeutic proteins. One example from Tremblay et al. (2011) demonstrated a high-yield of soybean agglutinin (SBA), a specific *N*-acetylgalactosamine-binding plant lectin, in potato tubers. The recombinant SBA retained its ability to induce hemagglutination, was similarly glycosylated to the native SBA and retained its binding specificity for *N*-acetylgalactosamine. In addition, the recombinant SBA was highly resistant to degradation in simulated gastric and intestinal fluid. One disadvantage of using transgenic potato for the production of antigenic proteins is the poor expression of the foreign proteins. In this regard, it is of interest to mention Youm et al. (2010) who examined the antibody response in mice orally immunized using various doses

of potato-derived major surface antigen of hepatitis B virus (ranging from 0.02 to 30 μg potato-derived antigen).

Approaches to increase the yield of recombinant protein expressed in transgenic plants include down regulation of native proteins within the tubers and targeting the protein to the cell secretory pathway. Tremblay et al. (2011) investigated whether minor interruption of starch biosynthesis can have a positive effect on tuber protein content and on tuber biomass. In order to increase the efficiency of the crop potato as a bioreactor, they used an RNAi approach to knock down ATP/ADP transporter in *Solanum tuberosum*. The authors identified a new line (riAATP1-10) with reduced starch accumulation, increased biomass yield and increased total protein levels. The potential of this line as a new bioreactor candidate was tested by expressing a human single-chain variable fragment (scFv) antibody. Protein expression in the riAATP1-10 line translated into a nearly fourfold increase in product yield. Kim et al. (2008e) also utilized the RNAi technology to knockdown various patatin isoforms in potato tubers for the development of a more efficient protein expression system. In an attempt to improve the yield, the activity and the stability of recombinant protein, Badri et al. (2009a, b) used protein targeting to produce transgenic potato lines expressing the protein bovine aprotinin targeted to the cytosol, the endoplasmic reticulum (ER) and the apoplast. Using a novel SELDI-TOF MS (surface-enhanced laser desorption ionization time-of-flight mass spectrometry) procedure, the authors were able to demonstrate that the recombinant protein targeted to the ER showed good accumulation levels but was processed in the ER compartment of plant cells (Badri et al. 2009a). In a subsequent paper, the authors used a combination of SELDI-TOF MS and 2-D gel analyses to demonstrate that aprotinin retention in the ER was associated with a decrease of leaf soluble protein content and down-regulation of proteins implicated in protein synthesis and maturation. This suggests nintended metabolic interference in transgenic plants (Badri et al. 2009b).

These data demonstrate the importance in plant-made vaccine design of considering also the possible effects of the foreign protein expression on native protein accumulation and endogenous metabolism of the host plant factory. Additional factors that could limit recombinant protein expression include processes such as silencing, premature polyadenylation, mRNA stability and improper codon usage. Mathew et al. (2011) showed the importance of eliminating spurious polyadenylation signals within the coding sequence of the Narita 104 virus capsid protein. Such an exercise increased foreign protein expression in potato plants. Finally, the variability in antigen expression could depend also on the different plant growth conditions used. For example, Mikschofsky et al. (2011) compared greenhouse and field production of potato-made foreign protein using potato expressing VP60 (structural capsid protein of the rabbit hemorrhagic disease virus), CTB and the marker protein NPTII and concluded that equal or higher expression levels with lower variability of foreign protein could be expected in the fields compared to greenhouse production.

4.6.3 Vaccine Production in Seed Crops

Oral administration requires about 100 times the amount of vaccine delivered through direct injection. Endotoxins that exist inside plants, such as solanine in potato tubers, also raise safety concerns, as they cannot be easily removed. In cases where further processing, such as cooking, is required, thermolabile proteins will be degraded in the process, further reducing the effective yield. Despite the challenges described, rice remains a competitive candidate for producing and delivering recombinant vaccines for two important reasons. First, rice does not contain endotoxins harmful to the human body. Second, localization of recombinant protein into seeds provides ideal storage due to low protease activity. Several vaccine antigens have been successfully produced in recombinant rice (Kuo et al. 2013). The envelope protein of Japanese encephalitis virus (JEV) has been expressed in rice leaves, and the maximum yield was 1.1–1.9 μg/mg of total protein (Wang et al. 2009). To examine the biological activity of the recombinant vaccine, mice were immunized using both *Escherichia coli* derived JEV envelope proteins and recombinant proteins from rice through oral administration. The rice-derived vaccine was able to elicit higher IgG and IgA responses compared to the recombinant protein derived from *E. coli*. Oral administration of *Chlamydophila psittaci* antigen, MOMP, fused to the B subunit of *E. coli* LTB produced in rice seeds has also successfully induced mouse immunity to mucosal disease (Zhang et al. 2009). Other vaccines were successfully produced for the intention of oral administration as uncooked rice powder, and biologically activities were examined through animal tests (Suzuki et al. 2011; Takagi et al. 2005). Transgenic rice expressing mouse dominant T cell epitope peptides of Cry j I and Cry j II allergens of Japanese cedar pollen was able to prevent the development of allergen-specific IgE and IgG responses (Takagi et al. 2005). Alternatively, transgenic rice expressing a fragment (pp. 45–145) of mite allergen (Der p 1) containing immunodominant human and mouse T cell epitopes successfully reduced the serum levels of allergen-specific IgE and IgG (Suzuki et al. 2011). It is worth noting that differences in mammalian and plant glycosylation have caused immunogenic response in both mice and human, indicating that further modification may be required for recombinant protein produced using rice expression systems (Kuo et al. 2013).

4.7 Options to Achieve Overproduction of Recombinant Proteins

Several techniques are available to increase the level of protein expression in plant cells (Egelkrout et al. 2012). Codon-optimization of the transgene, introduction of different regulatory elements and targeting of expressed proteins to special organelles are all factors that can enhance transcription and translational efficiencies and yield of recombinant proteins expressed in plant cells (Rigano et al. 2013). For

example, Jha et al. (2012) demonstrated that targeting recombinant human α1-proteinase inhibitor to different subcellular compartments in transgenic tomato plants could influence final yield, biological activity and in planta stability of the recombinant protein. In addition, the level of transgene expression in plants can be affected by the activity and specificity of the promoters. Recently, there has been a vast amount of effort invested in discovering various types of promoters. For example, in tomato several fruit-specific promoters have been recently identified and utilized (Estornell et al. 2009; Guillet et al. 2012; Hiwasa-Tanase et al. 2012; Kim et al. 2012). Targeting recombinant protein expression to the edible parts of the transgenic crop using fruit-specific promoters can be convenient for the production of oral plant-derived vaccines and to avoid non-specific alterations at whole plant levels. The identification of novel promoters expressed in specific tissues or during specific stages of development are proceeds of novel genomic resources and new high throughput sequencing methods such as RNA-Seq that allow evaluation of changes in expression of the transcriptome (Rigano et al. 2013).

The accumulation of recombinant proteins in plant cells is dependent not only on the synthesis of the products but also on its degradation (Rigano et al. 2013). Therefore, the potential of tomato cathepsin D inhibitor as a companion stabilizing agent for the protection of cytosol-targeted recombinant proteins in plants was recently investigated (Goulet et al. 2010). The authors demonstrated a proteome-wide, up-regulating effect of this inhibitor on endogenous leaf proteins in potato and a stabilizing effect in planta improving the accumulation of the cytosol-targeted heterologous protein human α1-antichymotrypsin.

An alternative to conventional nuclear transformation for the expression of high-yield recombinant protein in food crops is stable genetic transformation of plastids (Rigano et al. 2013). The integration and expression of transgenes in the plastid genome presents several advantages including high and stable production levels of foreign protein attainable; precise integration into the host plastid genome that relies on homologous recombination; absence of epigenetic effects; reduced risk of introducing transgenes into the food chain; and the possibility of co-expressing multiple transgenes from prokaryotic-like operons (Bock and Warzecha 2010; Cardi et al. 2010; Scotti et al. 2012). Previously, plastid transformation of species other than tobacco has been limited by low transformation frequencies and low transgene expression usually achieved in non-green plastid. However, today, protocols for stable plastid transformation in tomato and potato have been developed and made available to the scientific community (Ruf et al. 2001; Segretin et al. 2012; Valkov et al. 2011; Wurbs et al. 2007).

The work of Valkov et al. (2011) is noteworthy as it reports an improvement in potato transformation efficiency at levels similar to those obtained for tobacco. This result was achieved by the modification of the selection/regeneration procedure and by using novel vectors containing potato-flanking sequences for transgene integration by homologous recombination in the plastome. In addition, regulatory sequences that could increase protein expression in tomato chromoplasts and potato amyloplasts have been identified and tested (Caroca et al. 2012; Scotti et al. 2011; Valkov et al. 2011). Caroca et al. (2012) used tomato plastid transformation to test

a combination of promoters and 5′UTR for their potential to confer active gene expression in chromoplasts. The authors identified chimeric expression elements that trigger high-level protein accumulation in chromoplasts. The best-performing promoter-UTR combinations resulted in accumulation of the reporter protein GFP to 1 % of total cellular protein of the ripe tomato fruit, which is comparable to the GFP accumulation level achievable in chloroplast of green leaves. Tomato transplastomic plants have been used successfully to produce nutraceutical and biopharmaceutical proteins (Wurbs et al. 2007; Zhou et al. 2008). For example, Apel and Bock (2009) introduced lycopene β-cyclase genes from the eubacterium *Erwinia herbicola* and daffodil (*Narcissus pseudonarcissus*) into the tomato plastid genome in order to enhance carotenoid biosynthesis inducing lycopene-to-β-carotene conversion.

Hairy root cultures present an alternative system for producing useful pharmacological compounds in crops. In nature, hairy root is a disease of plant tissues infected by the soil bacterium *Agrobacterium rhizogenes*. This bacterium transfers specific genes (rol genes) from its endogenous root inducing plasmid (Ri plasmid) to alter the auxin/cytokinin perception of the host cells inducing neoplastic root and hair proliferation and growth. Hairy roots provide a genetically-stable transgenic tissue culture system that grows rapidly in simple phytohormone-free media (Rigano et al. 2013). With respect to production of foreign proteins, hairy roots are easily biocontained in a controlled in vitro environment; they can be scaled up to produce large amounts of biomass in industrial scale bio-reactors (Wilson 1997); and have the potential for rhizosecretion (Gaume et al. 2003). Hairy roots of many different plant species have been utilized to produce various recombinant proteins at varying yields and there have been studies into producing recombinant proteins in hairy roots from crop plants such as tomato and potato. For example, Sunil Kumar et al. (2006) optimized the generation and growth of potato hairy roots for the production of the hepatitis B surface antigen (HBsAg). De Guzman et al. (2011) reported production of the *Escherichia coli* B-subunit heat labile toxin antigen in tomato hairy root cultures (~10 μg/g blotted weight, BW) and compared the productivity against hairy roots of tobacco (~100 μg/g BW) and petunia (~100 μg/g BW). While tomato yielded the least amount of antigen overall, the antigen accumulation was reasonable when compared to other plant systems producing LT-B (Haq et al. 1995; Kosaki et al. 2008; Walmsley et al. 2003). Unfortunately, oral delivery of vaccine antigens within hairy root tissue has proven less efficient than other plant tissues perhaps due to the cell walls being too thick and not releasing enough antigens from the cells at optimal locations throughout the digestive tract (Pelosi et al. 2011, 2012). Secretion or purification of recombinant proteins from hairy roots may prove to be the more realistic approach for this platform. Lallemand et al. (2015) also focused on the analysis of extracellular proteolytic activities in two production systems: cell cultures and root-secretion (rhizosecretion), in *Arabidopsis thaliana* and *Nicotiana tabacum*.

4.8 Quality Aspects of Plant-Based Vaccines

For developing a plant-based expression system for vaccines, four criteria should be met. First, the major antigen must be sufficiently concentrated to allow for the recommended dose to be delivered in an amount of material that is easily consumed. For example, the standard dose for parenterally delivered hepatitis B vaccines varies from 5 to 40 μg, depending on the specific product, the age of the recipient and whether the recipient is immunocompromised (Streatfield 2005). It is anticipated that oral doses will need to be at least equivalent to parenteral doses, but may need to be up to three orders of magnitude greater in order to achieve comparable efficacy. Also, for the convenience of administration, the amount of material delivered orally as a vaccine should not exceed a few tens of grams. Thus, for a potential oral dose of 10 mg of hepatitis B major surface antigen to be delivered in 10 g of plant material, the recombinant protein must constitute 0.1 % of the material administered. Protein extraction procedures to concentrate the antigen from plant expression hosts are best avoided, because they add significant cost. However, for fresh tissue, some degree of dehydration is necessary to avoid rapid protein degradation following harvest, and this also serves to concentrate the antigen on a weight basis. Also, some inexpensive food processing technologies applied to plant tissues yield protein-rich fractions, and can thus serve to concentrate antigens up to tenfold. Thus, depending on the available processing options for the plant tissue chosen for antigen production, expression level targets should be at least 0.01–0.1 % weight.

A second criteria for a plant-based vaccine is that the antigen concentration should be uniform, allowing for even dosing of subjects. Some degree of processing will likely be required to achieve homogenization. Third, the plant material should be palatable, encouraging uptake. This rules out several plant expression systems such as tobacco and *Arabidopsis thaliana* used to show the feasibility of expressing antigens in plants (Tacket et al. 2000). Finally, the fourth criterion that should be met is that the antigen should be stable during prolonged storage of the vaccine at ambient temperatures, so avoiding the expense of a cold-storage chain. Grains are particularly suitable in this regard, although fresh tissue may be dehydrated to guard against antigen degradation (Kuo et al. 2013).

There have been several recent developments to improve the efficiency of downstream processing for the production of molecular pharming products. The need for quality and consistency in the manufacturing process has driven researchers to identify novel approaches to remove plant-specific contaminants and to develop models to facilitate purification. For example, flocculation (Buyel and Fischer 2014a) and heat precipitation (Buyel et al. 2014) have been shown to increase the efficiency of depth filtration during the purification of antibodies produced in tobacco leaves, and the filter train can also be optimized to remove plant-derived particulates more effectively (Buyel and Fischer 2014b). The behavior of the target protein and host cell proteins can be modeled to improve the overall efficiency of purification, including the use of quantitative structure activity relationship (QSAR) models to predict how host cell proteins behave during chromatography (Buyel et al. 2013).

4.9 Examples of Biopharma Products on and Close to the Market

Numerous companies have been involved in molecular farming over the last several years (Table 4.2). There are many products thought to be on and close to reaching the commercial market. Several of these proteins are normally derived from animal organs and, due to the possibility of animal pathogens being carried along with these proteins, there is a need for alternative low-cost supply. Table 4.2 lists these products.

4.9.1 Aprotinin

Aprotinin is a protein that inhibits serine proteases (including trypsin, chymotrypsin, kallikrein and pepsin). Under the trade name Trasylol and Apronexin, aprotinin was used as an injection to reduce bleeding during complex surgery, such as heart and liver surgery. Its main effect is the slowing down of fibrinolysis, the process that leads to the breakdown of blood clots. The aim of its use is to decrease the need for blood transfusions during surgery, as well as end-organ damage due to hypotension. Native aprotinin is extracted from bovine lungs. Zhong et al. (1999) reported the generation and characterization of transgenic maize lines producing recombinant aprotinin. Immature maize embryos were transformed with the aprotinin gene via particle bombardment. The recombinant aprotinin protein purified from transgenic maize seeds has biochemical and functional properties identical to its native protein. Prodigene Corporation (USA) and Sigma-Aldrich (USA) are marketing aprotinin (AproliZean) from maize and from a transgenic tobacco.

4.9.2 Avidin

Avidin is a glycoprotein found in avian, reptilian and amphibian egg white. It is used primarily as a diagnostic reagent. The protein is composed of four identical subunits, each 128 amino acids long. The usual source for commercial quantities of avidin is from chicken egg white but the resultant product is relatively expensive due to the cost of maintaining live animals. Hood et al. (1997) produced avidin in transgenic maize seed. The resultant avidin had properties almost identical to those of avidin from chicken egg white. Both the maize-derived and the chicken egg-derived avidin were glycosylated. While the avidin apoproteins were identical, the size of maize-derived avidin was slightly less than chicken egg-derived avidin due to a less complex glycosylation composition. This difference did not alter the binding activity of the mature protein. The production, purification and characterization of chicken egg-white avidin from transgenic maize seed was done by Kusnadi et al. (1998). This product is currently being sold by Sigma-Aldrich (USA).

Table 4.2 Molecular farming products thought to be close to or on the market

Product name	Companies/Organizations	Crop	Uses of product	References
AB	Large Scale Biology	*Nicotiana* sp.	Cancer vaccine	Spok and Karner (2008)
A-Herpes MAb (anti-HSV antibody)	Epicyte Pharmaceutical	*Zea mays*	Prevention of herpes transmission	Spok and Karner (2008)
Albumin-DX LR	Ventria (InVitria)	*Oryza sativa*	Uses for stem cell and regenerative medicine cell lines	Fischer et al. (2014)
Apo AI(Milano)	SemBioSys	*Carthamus tinctorius*	Key factor in eradicating coronary heart disease	Spok and Karner (2008)
Aprotinin (serine protease inhibitor)	ProdiGene, Large Scale Biology	*Z. mays*	Treatment of profuse incoercible bleeding during rhytidectomy	Zhong et al. (1999) and Pogue et al. (2010)
			In cardiac surgery	
Avidin (immunological reagent)	ProdiGene	*Z. mays*	Uses in clinical applications	Hood et al. (1997)
Brazzein (Natural protein sweetener)	ProdiGene	*Z. mays*	Treatments for obesity, diabetes and malabsorption syndromes	Lamphear et al. (2005)
CaroRx™	Planet Biotechnology	*Nicotiana* sp.	Prevention tooth decay caused by bacterial infection	Ma et al. (1998)
Cellastim, Optibumin (recombinant human serum albumin)	Ventria (InVitria)	*O. sativa*	Suited as a cell culture media supplement for stem cells, primary cells, CHO cells and Vero cells	Fischer et al. (2014)
			Uses in stabilizing biomedical products like viral vaccines, cell therapies, therapeutic proteins, bioactive hydrophobic compounds, and other therapeutic applications	
Collagen (Fibrous protein)	ProdiGene, Medicago, Meristem Therapeutics	*Z. mays*	Anti-aging skincare treatments	Spok and Karner (2008) and Breyer et al. (2009)

(continued)

Table 4.2 (continued)

Product name	Companies/Organizations	Crop	Uses of product	References
DoxoRx™	Planet Biotechnology	*Nicotiana* sp.	For chemotherapy drug-induced alopecia	Spok and Karner (2008)
Escherichia coli heatlabile toxin (LT)	Prodigene Inc., Arntzen group	*Solanum tuberosum*, *Z. mays*	Treatment of diarrhea	Tacket et al. (1998) and Chikwamba et al. (2002)
ELELYSO, PRX-112 [glucocerebrosidase (GCD) enzyme]	Protalix Biotherapeutics	*Daucus carota* cell culture	Treatment of gaucher disease	Shaaltiel et al. (2007)
Gastric lipase (recombinant dog gastric lipase)	Meristem Therapeutics	*Z. mays*	Treatment of cystic fibrosis and pancreatitis	Zhong and Glalz (2006)
GUS	ProdiGene	*Z. mays*	Uses as diagnostic reagent	Witcher et al. (1998)
H1N1, H5N1 vaccine (influenza vaccines)	Medicago	*Nicotiana benthamiana*	Treatment of influenza flu	D'Aoust et al. (2008)
Hepatitis B virus surface antigen	Arntzen group, Thomas Jefferson University/Polish Academy of Sciences	*S. tuberosum*, *Lupinus*, *Lactuca sativa*	Vaccine for hepatitis B	Richter et al. (2000) and Kapusta et al. (1999)
Human Activin A, Activin B	ORF Genetics, Agrenvec	*Nicotiana* sp.	Uses for hematopoiesis, immune response, neural cell differentiation, wound repair and morphogenesis	www.orfgenetics.com; www.agrenvec.com
Human BAFF (B lymphocyte activating factor)	Agrenvec	*N. benthamiana*	Enhances B cell and regulate peripheric B cell	www.agrenvec.com
			Vital homeostatic cytokine for B cells that helps regulate both innate and adaptive immune responses	

Human BMP-2	ORF Genetics	*N. tabacum*	Uses for embryonic development, including induction of bone and cartilage growth and regeneration, cardiac cell differentiation and epithelial to mesenchymal transition	www.orfgenetics.com
Human BMP-7	Agrenvec	*N. benthamiana*	Promotes new bone formation and nephron development	www.agrenvec.com
			Inhibits the branching of prostate epithelium	
			Antagonizes epithelialmesenchymal transition (EMT)	
Human EGF (epidermal growth factor)	ORF Genetics, Agrenvec	*Hordeum vulgare*, *N. benthamiana*	Stimulates the proliferation of various epidermal and epithelial cells	www.orfgenetics.com; www.agrenvec.com
Human FGF basic 146 (bFGF or FGF2)	ORF Genetics	*H. vulgare*	Multifunctional proteins involve in angiogenesis, wound healing, and embryonic development	www.orfgenetics.com
Human Flt3-ligand (FMS related tyrosine kinase 3 ligand, flk-2 ligand)	ORF Genetics, Agrenvec	*H. vulgare*, *N. benthamiana*	Induces proliferation of early hematopoietic cells	Erlendsson et al. (2010); www.agrenvec.com
Human Follistatin	Agrenvec	*N. benthamiana*	Suppresses synthesis and secretion of follicle-stimulating hormone (FSH) from the pituitary gland	www.agrenvec.com
Human G-CSF (granulocyte colony-stimulating factor, filgrastim, pegfilgrastim or lenograstim)	ORF Genetics	*H. vulgare*	Treatment of leukopenia following cancer therapy and bone marrow transplantation	www.orfgenetics.com

(continued)

Table 4.2 (continued)

Product name	Companies/Organizations	Crop	Uses of product	References
Human GDNF (glial cell line derived neurotrophic factor)	ORF Genetics	*H. vulgare*	Treatment of Parkinson's disease and spinal cord injuries	www.orfgenetics.com
Human GM-CSF (granulocyte macrophage colony-stimulating factor)	ORF Genetics, Agrenvec	*O. sativa* suspension culture, *N. benthamiana*	Uses for reconstitution of the hematopoietic system following cancer therapy or bone marrow transplantation	www.orfgenetics.com; www.agrenvec.com
Human growth hormone	Agrenvec	*N. benthamiana*	Uses for growth control	Gils et al. (2005)
Human IFN alpha	Agrenvec	*N. benthamiana*	Uses as antiviral, antiproliferative and in immunomodulatory activities	www.agrenvec.com
Human IFN gamma	ORF Genetics	*H. vulgare*	Uses as antiviral and antiparasitic activities	www.orfgenetics.com
			Uses for immunoregulatory and proinflammatory activities	
Human IGF-I (insulin-like growth factor I)	Agrenvec	*N. benthamiana*	Uses for cellular energy metabolism and in growth and development	www.agrenvec.com
Human IL-2 (Interleukin 2)	ORF Genetics	*H. vulgare*	Promotes growth and differentiation of various cells of the immune system	www.orfgenetics.com
			Essential for proper immune response, and important factor in the natural suppression of autoimmunity	
			Uses as an anti-tumor agent in cancer therapy	

Human IL-3 (Interleukin-3, Hematopoietic growth factor, mast cell growth factor)	ORF Genetics, Agrenvec	*H. vulgare*, *N. benthamiana*	Uses for proliferation and differentiation of various hematopietic cell types, including pluripotent stem cells	www.orfgenetics.com; www.agrenvec.com
Human IL-4 (Interleukin 4, B cell stimulatory factor)	ORF Genetics, Agrenvec	*H. vulgare*, *N. benthamiana*	Regulates the differentiation of naive $CD4^+$ T cells into helper Th2 cells, and regulation of B cells IgE and IgG1 production	www.orfgenetics.com; www.agrenvec.com
Human IL-6	ORF Genetics, Agrenvec	*H. vulgare*, *N. benthamiana*	Regulates the immune and inflammatory responses	www.orfgenetics.com; Nausch et al. (2012)
Human IL- 7	Agrenvec	*N. benthamiana*	Stimulates the adaptive immune response	www.agrenvec.com
Human IL- 10	Agrenvec	*N. benthamiana*	Uses as anti-inflammatory	Bortesi et al. (2009)
Human IL- 12 subunit p40	Agrenvec	*N. benthamiana*	Major factor for the development of cellular immunity	www.agrenvec.com
Human intrinsic factor	Cobento Biotech A/S	*S. tuberosum*, *Arabidopsis*	Uses in vitamin B12 deficiency	Fedosov et al. (2003)
Human myostatin (GDF8, MSTN)	Agrenvec	*N. benthamiana*	Induces muscle hypertrophy as well as enhanced glucose utilization and insulin sensitivity and a reduction in overall fat mass	www.agrenvec.com
Human keratinocyte Growth factor (KGF, FGF7, HBGF-7)	Greenovation, ORF Genetics	*H. vulgare*, *N. benthamiana*	Uses for homeostasis, response to injury, and tissue repair	www.orfgenetics.com; www.agrenvec.com
			Uses for embryonic development and postnatal growth	
Human LIF (leukemia inhibitory factor), Mouse LIF	ORF Genetics, Ventria (InVitria)	*H. vulgare*, *O. sativa*	Inhibits the proliferation of myeloid leukemia cells and induce their differentiation into macrophages	www.orfgenetics.com; Youngblood et al. (2014)

(continued)

Table 4.2 (continued)

Product name	Companies/Organizations	Crop	Uses of product	References
Human liver X Receptor beta	Agrenvec	*N. benthamiana*	Role in cholesterol, lipid, and carbohydrate metabolism	www.agrenvec.com
Human M-CSF (macrophage colony stimulating factor, colony stimulating factor 1, CSF-1)	ORF Genetics	*H. vulgare*	Enhances antibody-dependent cell-mediated cytotoxity	www.orfgenetics.com
			Enhances marophage killing of tumor cells and microorganisms	
			Regulates the release of cytokines and inhibits bone resorption by osteoclasts	
Human SCF (stem cell factor, c-kit ligand, mast cell growth factor, steel factor), Mouse SCF	ORF Genetics	*H. vulgare*	Promotes the survival, proliferation and differentiation of both myeloid and lymphoid hematopoietic progenitors in bone marrow culture	www.orfgenetics.com
			Stimulates the growth and activation of mast cells and eosinophils	
Human sRANKL	Agrenvec	*N. benthamiana*	Role in bone remodeling and disorders of mineral metabolism	www.agrenvec.com
Human TFPI-2 Domain 1	Agrenvec	*N. benthamiana*	Inhibit Endothelial Cell Matrix (ECM) proteases essential for angiogenesis and metastasis	Williams et al. (2014)
Human TGF β-2, Human TGF β-3	Agrenvec	*N. benthamiana*	Involve in physiological processes as embryogenesis, tissue remodeling and wound healing	www.agrenvec.com

Human TIMP-2	Agrenvec	*N. benthamiana*	Play an important role in wound healing, apoptosis, bone elongation, embryo development, uterine involution, angiogenesis, and tissue remodeling	www.agrenvec.com
			Uses in diseases such as multiple sclerosis, Alzheimer's, malignant gliomas, lupus, arthritis, periodontitis, glomerulonephritis, atherosclerosis, tissue ulceration, and in cancer cell invasion and metastasis	
Human TNF alpha (tumor necrosis factor, alpha)	ORF Genetics	*H. vulgare*	Regulates immune cells	www.orfgenetics.com
			Cytotoxic effect to various tumor cells	
			Causes inflammation, cellular proliferation and mediates resistance to bacterial infections	
Human TRX (Thioredoxin)	Agrenvec	*N. benthamiana*	Anti-oxidant properties	www.agrenvec.com
			Involves in several redox-dependent processes such as gene expression, signal transduction, cell growth, apoptosis and interacting with various kinds of target molecules	
Human VEGF165 (vascular endothelial growth factor)	ORF Genetics, Agrenvec	*H. vulgare*, *N. benthamiana*	Promotes endothelial proliferation and survival	www.orfgenetics.com; www.agrenvec.com
			Uses for angiogenesis, vasculogenesis and inhibits apoptosis	
Insulin	SemBioSys	*Carthamus tinctorius*	For treatment of diabetes	Spok and Karner (2008)

(continued)

Table 4.2 (continued)

Product name	Companies/Organizations	Crop	Uses of product	References
Lactoferrin (Glycoprotein)	Meristem Therapeutics	*Z. mays*	For iron deficiency and acute diarrhea	Samyn-Petit et al. (2001)
Lacromin (recombinant lactoferrin)	Ventria (InVitria)	*O. sativa*	Suited as a cell culture media supplement for stem cells, primary cells, CHO cells and HEK293 cells	Nandi et al. (2005)
Lysobac (recombinant human lysozyme)	Ventria (InVitria)	*O. sativa*	Uses as a preservative in foods and beverages, diagnostic applications, bioprocessing and life science research	Nandi et al. (2005)
			Treatment of suppurative infections in diabetics	
Norwalk virus capsid protein	Arntzen group	*S. tuberosum*	Vaccine for Norwalk virus infection	Tacket et al. (2000)
Optiferrin (recombinant human transferrin)	Ventria (InVitria)	*O. sativa*	Uses as iron carrier	Zhang et al. (2010)
PRX-102 (alpha-Galactosidase-A protein)	Protalix Biotherapeutics	*Daucus carota* cell culture	Treatment of fabry disease	Kizhner et al. (2015)
PRX-106 [anti-TNF (tumor, necrosis factor)]	Protalix Biotherapeutics	*D. carota* cell culture	Uses as anti-cancer	www.protalix.com
PRX-110 [human deoxyribonuclease I (DNase I)]	Protalix Biotherapeutics	*D. carota* cell culture	Treatment of cystic fibrosis	Shaaltiel et al. (2013)

Rabies glycoprotein	Medicago,	*N. benthamiana*, *Spinacia oleracea*	Treatment of rabies	D'aoust et al. (2012)
				Yusibov et al. (2002)
RhinoRx™	Planet Biotechnology	*Nicotiana* sp.	For rhinovirus colds	Spok and Karner (2008)
Rotavirus vaccine	Medicago	*N. benthamiana*	Treatment of diarrhea in infants and young children	Spok and Karner (2008)
TGEV edible vaccine	ProdiGene	*Z. mays*	TGEV vaccine in swine	–
Trypsin	ProdiGene	*Z. mays*	Treatment of malignant disease	Woodard et al. (2003)
scFv antibody fragments	Large Scale Biology Corp	*Nicotiana* sp.	Non-Hodgkin's lymphoma	Fiedler et al. (1997)

4.9.3 Collagen

Collagen is a group of naturally-occurring proteins. In nature, it is found exclusively in animals, especially in the flesh and connective tissues of mammals. It is the main component of connective tissue, and is the most abundant protein in mammals. Based on their structural roles and compatibility within the body, collagen and gelatin are commonly used biomaterials in the medical, pharmaceutical and cosmetic industries. Collagen is used commercially in the areas of bone grafts, corneal implants, drug delivery, incontinence, tissue engineering and as a viscoelastic supplement. Gelatin is used in the stabilization and delivery of vaccines and drugs, in capsules and soft gels, nutraceuticals and as plasma expanders. The first report of human collagen produced in plants was by Ruggiero et al. (2000). They used tobacco plants as a novel expression system for the production of human homotrimeric collagen. Fibrillar collagen cDNAs was inserted into tobacco using *Agrobacterium*. Large amounts of recombinant collagen were purified from field-grown plant material. The data suggest that plants are a valuable alternative for the recombinant production of collagen for various medical and scientific purposes.

4.9.4 Edible Vaccines

Vaccine delivery through crops could have significant benefits, most notably in the developing world. Vaccines could be grown from seed and then freely distributed without the need for trained medical staff at any stage. Implementation of such schemes would probably require initially-high expenses in terms of education and training, data monitor points out, but thereafter would be relatively cheap. Plant-based vaccines are now edging closer to commercialization. Prodigene Inc. is currently leading the way in developing plant vaccine technology. In the last several years, a novel approach for developing improved mucosal subunit vaccines has emerged by exploiting the use of genetically-modified plants. It has been demonstrated that plant-derived antigens are functionally similar to conventional vaccines and can induce neutralizing antibodies in mammalian hosts. Using genetically engineered plants for the production of immunogenic peptides also provides a new approach for the delivery of a plant-based subunit vaccine, i.e. oral delivery, provided these immunogenic peptides are expressed in an edible plant part, such as the fruit. Thus, food crops can play a significant new role in promoting human health by serving as vehicles for both production and delivery of vaccines (Korban et al. 2002). Charles Arntzen, Arizona State University, has pioneered the field with work on hepatitis B and LT-B in tobacco plants and potato tubers. Vaccines administered using needles do not usually give a good immune response in the mucosal tissues of the vaccine recipient. The laboratory of William Langridge at Loma Linda University, California, is developing multi-component mucosal subunit vaccines and active in developing edible vaccines against diabetes and cholera. Arakawa et al. (1998, 2001) have generated transgenic potato plants that synthesize human insulin, a major insulin-dependent diabetes mellitus autoantigen, at levels up to

0.05 % of total soluble protein. Korban et al. (2002) reported a plant-based oral vaccine against respiratory syncytial virus (RSV) in tomato fruit and are moving the product into apple. Dry grains (maize, rice, wheat) may prove to be a superior delivery system for edible vaccines since the antigen will remain at a constant level for an extended period of time without refrigeration (Streatfield 2002). Researchers have to solve the existing disadvantages such as low yields, immunogenicity, accumulation and stability of the transproteins, and obtaining glycosylation; processes that are normally observed in humans.

4.9.5 β-Glucuronidase

β-glucuronidases are members of the glycosidase family of enzymes. GUS is a homotetrameric hydrolase that cleaves b-linked terminal glucuronic acids in mono and oligo-saccharides and phenols. The protein is composed of four identical subunits, each 128 amino acids long. It is used primarily as a diagnostic reagent as visual marker in transgenic plant research. The usual source for commercial quantities of GUS is chicken egg. It was first produced in transgenic maize (Kusnadi et al. 1998) and its properties were compared with GUS extracted from *Escherichia coli*, the original source of the protein. While many other investigators have demonstrated the expression of GUS as a scoreable marker, Witcher et al. (1998) have generated transgenic maize seed containing GUS for commercial production. This is one of the first cases where a detailed characterization of the transgenic plants and the protein were performed which are necessary to use this as a commercial source of GUS. Maize-derived GUS has been marketed by Sigma-Aldrich (USA).

4.9.6 Human Gastric Lipase

Human gastric lipase is involved in exocrine pancreatic insufficiency (EPI). EPI is the inability to properly digest food due to a lack of digestive enzymes made by the pancreas. The current supply of gastric lipase is from bovine pancreatic tissue. A recombinant dog gastric lipase with therapeutic potential for the treatment of EPI was expressed in transgenic tobacco plants (Gruber et al. 2001). Meristem Therapeutics is advancing a maize-derived mammalian lipase through clinical trials.

4.9.7 Human Lactoferrin

Lactoferrin is secreted in milk, tears and bile. It is elevated by inflammation and some cancers. It has been suggested to have a number of functional properties including anti-cancer, immunomodulation, anti- microbial, anti-viral, toxin binding

properties (Legrand et al. 2003). In the work done by Samyn Petit et al. (2001), the human lactoferrin coding sequence was inserted into the pUC18 plasmid under control of the wheat glutenin promoter. Maize was stably transformed and recombinant lactoferrin was purified. N-glycosylation sites of recombinant lactoferrin are mainly substituted by typical plant paucimannose-type glycans, with beta1,2-xylose and alpha1,3-linked fucose at the proximal N-acetylglucosamine, and that complex-type glycans with Lewis(a) determinants are not present in maize recombinant lactoferrin. Studies at the Baylor School of Medicine, Texas USA, were carried out relative to the cloning of lactoferrin and lactoferrin peptides and the expression of recombinate lactoferrin by fungi and bacteria (Conneely et al. 1996a, b, c). It is claimed that the verified cDNA sequence for human, bovine and porcine lactoferrin protein have been used to prepare lactoferrin for therapeutic and nutritional applications. Regions of the cDNA, such as the iron binding sites can be used to produce peptide products. The conversion of these concepts to commercial practice could be competitive with the current production of lactoferrin from whey. Each natural lactoferrin molecule has two iron-binding domains, which reversibly bind iron. Recombinant human lactoferrin has been produced in *Aspergillus* sp. from which good levels for pharmaceutical have been obtained (Conneely et al. 2001). Lactoferrin was first reportedly produced in tobacco suspension cells by Mitra and Zhang (1994). A suspension tobacco cell line was transformed to express human lactoferrin. The transgenic calli produced a protein that was significantly smaller than the full-length lactoferrin protein. Total protein extracts made from transgenic tobacco callus exhibited much higher antibacterial activity than commercially available purified lactoferrin.

4.9.8 Trypsin

Bovine trypsin is a protease that is used in a variety of commercial applications including the digesting or processing of other proteins, including some therapeutic proteins. Trypsin is an aggressive proteolytic enzyme. Recombinant trypsin has been produced in a number of systems, including cell culture, bacteria and yeast. But no system is commercially viable on a large scale. The biopharmaceutical industry is trying to eliminate animal-derived proteins from manufacturing processes due to the possible contamination of these products by human pathogens. Maize-derived trypsin has a significant market potential. The availability of maize-derived bovine trypsin helps to supply the growing market for animal-free reagents. This eliminates animal-source materials and reduces the fears of contamination of products by mammalian viruses. Expression of this enzyme at commercially-viable levels in maize was possible only by expressing the enzyme in an inactive zymogen form. Although the zymogen gene was put into plants, the active enzyme was recovered in extracts from maize seeds (Woodard et al. 2003). Maize derived trypsin (TrypZean) is the first large-scale protein product from transgenic plant technology. Maize-derived trypsin has been marketed by Sigma-Aldrich (USA).

4.10 Conservation of Developed Cell Lines

Cryopreservation refers to storage of living cells, tissues or organs at ultra-low temperatures, usually that of liquid nitrogen (−196 °C). When stored at such a low temperature, cellular divisions and metabolic processes of the living cells arrest and therefore, plant materials can be conserved for an indefinite period of time, while maximally maintaining their genetic stability (Xu et al. 2011). Furthermore, cryopreservation occupies little space, eliminates contamination and demands only maintenance. Therefore, cryopreservation has been considered an ideal means for safe and long-term storage of plant germplasm (Wang et al. 2012a). Safe maintenance of transgenic materials has been a serious problem, especially in developing countries. Transgenic materials are frequently maintained either in vitro or in vivo before being analyzed, evaluated and finally released for commercial production. Maintenance of transgenic materials by repeated subculture is time consuming, has high labor cost and may lead to culture loss due to contamination or human error. More importantly, in vitro or in vivo maintenance may create an extra risk of transgene loss or gene flow (Wang et al. 2012b). Therefore, establishment of safe and long-term preservation techniques for transgenic materials, which is capable of ensuring genetic integrity of transgenes and avoiding gene flow, is of significant importance. Successful cryopreservations have been reported for transgenic cell suspensions that expressed human serum albumin (HAS) in *Nicotiana tabacum* (Schmale et al. 2006), *Escherichia coli* heat labile enterotoxin (LT) protein in *N. tabacum* (Van Eck and Keen 2009) and hCTlA4Ig in *Oryza sativa* (Cho et al. 2007). All results obtained so far indicate that cryopreservation does not affect expression of foreign genes in transgenic materials and the productive ability of cryopreserved cells containing recombinant proteins was similar to that in non-cryopreserved cultures, thus allowing transgenic materials to be stored in a safe manner before being analyzed, evaluated and established as stable seed stocks for commercial production of homologous proteins (Wang et al. 2012b).

4.11 Future Prospects and Biosafety Challenges

After about two decades production of recombinant proteins in plants, only recently has the focus shifted away from technical and basic studies to a serious consideration of the requirements for sustainable productivity and the biosafety regulatory approval of pharmaceutical products (Sparrow et al. 2013). The manufacturing and clinical development of plant-derived pharmaceutical proteins falls under the same safety and good manufacturing practices (GMP) regulations covering drugs from all other sources (Jouzani and Tohidfar 2013). Only recombinant proteins produced by plant cell suspensions in the bioreactor systems may practically observe the GMP guidelines; other plant systems are needed to improve new GMP and biosafety standards and regulations (Fischer et al. 2012).

The objective of risk assessment is to identify and evaluate on a case-by-case basis potentially adverse effects of a genetically modified (GM) plant on the environment and human health. Through this approach, the GM plant is compared with its non-GM parent (substantial equivalence) having a safe use history and familiarity for the environment, in order to identify differences (Jouzani and Tohidfar 2013). Risk assessment is performed principally according to the following steps, including problem formulation and hazard identification, hazard characterization, exposure assessment, risk characterization, identification of risk management and communication strategies, and, finally, overall risk evaluation and conclusions. The risk assessment finally leads to a conclusion as to whether the overall health and environmental impact of the GM plant can be accepted or not (Breyer et al. 2012; EFSA 2009; Jouzani 2012). Similar to all genetically-modified plants, those intended for molecular farming must go through a complete risk assessment before they can be used in the field. However, in addition to the risk assessment framework of GM plants used as food/feed or processing, plant molecular farming (PMF) raises new questions and concerns that might trigger a need for specific biosafety considerations due to the nature of the recombinant genes used (Sparrow et al. 2013; Valkova et al. 2013).

The risk of co-mingling and contamination of transgenic plants used as a source of PMF with other agriculturally important crops could be reduced by use of non-food/feed crops as a source of PMF, production of recombinant proteins by cell suspension cultures in bioreactors, strict physical agronomic confinement and containment strategies for food/feed crops, post-harvest field monitoring and cleaning, use of late maturing or early maturing cultivars or planting at different periods to ensure harvesting at different periods from other crops intended for food/feed and processing (Obembe et al. 2011; Spok et al. 2008). Vertical gene flow or gene flow by plant sexual reproduction is the most important form of transgene pollution and occurs commonly via the dispersal of transgenic pollen. Plants for molecular farming should be chosen with the minimum possible gene flow and minimum seed production (Valkova et al. 2013). The biosafety strategies to prevent vertical gene flow include the use of closed isolated physical containment facilities (greenhouses, glasshouses, hydroponics and plant cell suspension cultures) and biological containment (self-pollinating species; cleistogamous lines). Other strategies to prevent vertical gene flow include chloroplast transformation, cytoplasmic male-sterile transgenic plants, sexually incompatible crop with wild relatives, non-germinating seeds or nonsprouting tubers/bulbs, engineered parthenocarpy and apomixes, transgene excision, tissue-specific expression of the transgene and the use of inducible promoters (Jouzani 2012; Obembe et al. 2011; Schillberg et al. 2013; Valkova et al. 2013). Horizontal gene transfer commonly is known as exchange of genetic material between sexually incompatible species belonging to different taxonomic groups and is often observed between bacteria (Valkova et al. 2013). The risk of horizontal gene transfer (especially, antibiotic resistance genes) from plants to microbes is generally believed to be extremely low, as there has been no report of such an incidence to date, and, in addition, it is important to note that loads of microorganisms, such as symbiotic, pathogenic, endophytic and ectophytic bacteria and fungi with antibiotic resistant genes, are naturally harbored by plants (Obembe et al. 2011).

The use of food crops as production systems for pharmaceuticals or industrial compounds is a controversial issue. There are several arguments in favor of using food crops for PMF (Hennegan et al. 2005; Sparrow et al. 2007; Streatfield et al. 2003) and all the biopharmed products currently on the market are produced via maize production platforms. However, many people are concerned about the risks such GM crops would pose in case they would inadvertently enter the food or feed chain. A classic example of such accidental contamination is the ProdiGene incident in 2002 (Breyer et al. 2009). Following a standard crop rotation practice, farmers had planted conventional soybeans for human consumption on land previously used to test in the field the ProdiGene GM maize that produces trypsin, a pancreatic serine protease. As a result, maize seed left from the transgenic crop grew in the soybean fields. Accidental contamination of the food chain relates not only to safety aspects but creates new questions about the financial liability of farmers and agricultural and food production industries. In the ProdiGene case, the company accepted a civil penalty of USD 250,000 and also covered the cost of destroying the soybean crop and the clean up steps that followed (Fox 2003).

4.12 Conclusions and Prospects

For molecular farming of pharmaceutical proteins, plants have advantages over traditional systems. These consist of low cost of production (Table 4.3), rapid scalability, absence of human pathogens, and the ability to fold and assemble complex proteins accurately. Therefore plants might be better than other production systems and it should be possible to make pharmaceuticals available to everyone who needs them, at a cost that everyone can afford. For the biotechnology and drug industry, bio-farming offers financial and health profit once the present phase of product development reaches the commercialization stage. However, for these benefits to be fully realized, the central issue of risk to the food industry and the environment is an important factor. A combination of strong and adaptable regulatory oversight with

Table 4.3 Comparison of production and effective cost for three countries and two presentations

	Korea or India	United States		Korea		India	
	Yeast-derived	Plant-derived		Plant-derived		Plant-derived	
Countries	Ten dose vials	Single dose packet	Ten dose packet	Single dose packet	Ten dose packet	Single dose packet	Ten dose packet
Cost (USD)	0.27	0.15	0.06	0.09	0.04	0.075	0.03
Effective cost (USD)	0.42	0.16	0.08	0.10	0.05	0.08	0.04
Percent saving for plant-derived vaccine against yeast-derived	–	62	81	76	88	81	90

Source: Arntzen et al. (2006)

technological solutions are required if the goals of realizing the full potential of plant molecular farming are to be met. Above all, plants need to be viewed as an option among many for manufacturing therapeutic proteins. Attention is now shifting from basic research towards commercial utilization, and molecular farming is attaining the phase at which it could challenge established production technologies that exploit bacteria, yeast and cultured mammalian cells. In this review, we highlight current advancements in molecular farming and the possibility for commercial drug development and production.

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Part II
Forage and Tree Traits

Chapter 5
Forages: Ecology, Breeding Objectives and Procedures

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Abstract Forages are integral components of grassland and pasture agro-ecosystem. They are the major source of feed and nutrition for livestock. As primary producers, they are converted by livestock into secondary production in the food chain. Forage breeding is a complex process involving plant morpho-physiological aspects (perenniality, mode of reproduction, mating systems) and aberrant plant-environment correlations affecting plant performance under various sward conditions. The ultimate aim of forage breeding is to develop cultivars with high and sustainable herbage yield under various management systems. It also encompasses development of cultivars with beneficial impacts on ecosystem functions, animal growth and health. This chapter addresses challenges for forage producers and breeders due to rapidly diminishing grassland areas and the impact on the biodiversity of grassland ecosystems and their productivity. Approaches to conserve genetic diversity and utilize forage genetic resources in an efficient way as well as breeding procedures practical use in selected forage crops are discussed.

Keywords Forage • Persistence • Polyploidy • Swards • Recurrent selection

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5.1 Introduction

Forage may be defined as referring to any herbaceous plant or part of a plant that is consumed by animals, while forage or fodder crops are grazed by animals or harvested as green chop, hay, silage or soiling (Allen et al. 2011). Animals are indispensable components in the food chain system. They feed on plant materials that are not directly consumed by humans. Forages are the major source of feed and nutrition for livestock. As primary producers, they are converted by livestock into secondary production of the food chain, including meat, milk, wool, skins, fur and farm yard manure. Consequently, forages may provide higher returns to producers than other grain crops such as wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.) and maize (*Zea mays* L.). Cultivation of forage legumes increases soil fertility by adding fixed nitrogen and improving soil properties, i.e. organic matter content, availability of phosphates, reduced susceptibility to wind and water erosion. Rotation of annual forages with grain crops results in a higher grain yield following forages, shift in weed population, improved C-sequestration, reduced NO_3 leaching in the soil profile and provides habitat for wildlife (Entz et al. 2002).

Forages have significantly contributed to the economies of many countries. The value of forages and their products in international trade was 619 million mt worth USD 365 million (FAO 2011). Economies of some countries, i.e. New Zealand, are closely dependent on dairy and livestock industry. New Zealand's export value of meat and dairy products was USD 17 billion in 2012. In addition to their direct contribution to the livestock industry, forage legumes, i.e. white clover (*Trifolium repens* L.), may indirectly contribute to the economy through nitrogen fixation, honey and seed production. It has been estimated that white clover can fix 1.57 million mt of nitrogen in New Zealand, worth USD 1.49 billion compared with USD 8.14 billion worth of gross agriculture production from New Zealand's pastoral sector (New Zealand Agriculture Statistics 2005). White clover seed and honey production can bring USD 25 million and USD 20 million annually, respectively (New Zealand Year Book 2000). In the USA, total earnings from the export of forage seed was USD 34 million, USD 12.2 billion from sale of hay, forage legumes provided nitrogen worth of USD 98 per hectare, and indirect benefits from the beef, calves and dairy products were worth of USD 66 billion (Economic Research Services 2010; National Agriculture Statistics Services 2010).

A wide range of annual and perennial forage crops have been used in various forage production systems such as conventional arable, integrated arable, ecological arable, conventional forage, integrated forage and ecological forage systems (Eltun et al. 2002). These systems include an array of plant species such as grasses, legumes, trees and cacti, and may also include other components, i.e. roots and crop residues. These forage resources differ in yield and quality that are determined by plant species and environmental conditions. Generally, grasses are high in complex carbohydrates while legumes have high protein content. Each forage crop species has its own limitations and diversified breeding objectives. Among various forage systems, integrated forage systems followed by ecological forage systems have positive impacts on the ecosystem functions (Eltun et al. 2002).

Forage breeding is a complex process involving plant morpho-physiological aspects (perenniality, mode of reproduction, mating systems) and aberrant plant-environment correlations affecting plant performance under various sward conditions. The ultimate aim of forage breeding is to develop cultivars with high and sustainable herbage yield under various management systems. Moreover, it also encompasses development of cultivars with beneficial impacts on animal growth and health. A forage cultivar should contain a high proportion of easily digestible matter and a low concentration of toxic components that have negative impact on animal health. Historically, plant breeding has been very successful in delivering high quality forage crops for animal production. There are numerous examples of breeding forage crops with reduced concentrations of specific toxins such as HCN and formononetin, and increased forage quality. For example, selection of red clover for low formononetin content resulted in a 2.2 % increase in forage quality. Genetic gain may be one measure of the effectiveness of diversified forage breeding objectives such as high forage production and seed yield or better adaptability and persistence. However, genetic gains resulting from selection for these traits have been found relatively small and vary by geographical location (Woodfield 1999). Genetic gains in various forage species reach the range of 0.25–1.18 % annually (Woodfield 1999) and are usually lower in perennial than annual forages. Genetic gains in diploid species are typically higher than polyploid species. Woodfield and Caradus (1994) reported a 1.44 g m^{-2} increase in forage dry matter (DM) yield of white clover and a 0.14 % increase in white clover proportion of the sward biomass annually. In case of bromegrass (*Bromus* sp.), forage DM yield has increased by 0.54 Mg ha^{-1} (7.2 %), while resistances to brown leaf spot disease increased by 0.21 unit $year^{-1}$. Genetic gain of 4 % $decade^{-1}$ in DM yield has been reported for perennial ryegrass (Casler et al. 2000). In perennial ryegrass (*Lolium perenne* L.), the rate of genetic gain obtained through hybridization and recurrent selection can be maintained in ten subsequent generations. The genetic gain in nitrogen fixation efficiency of forage legumes has been increasing by 1.2 % annually. The increase in nitrogen fixation efficiency has been correlated with the selection for higher forage yield per se. Improvement in the nutritional value of forages started in the 1960s along with the advancement in analytical chemistry. Genetic improvement in forage digestibility estimated through in vitro dry matter digestibility (IVDM) has been documented in diverse forage species such as legumes and cool- and warm-season annual and perennial grasses (Casler and Vogel 1999). The IVDM has been shown to increase by 8–45 g kg^{-1} $cycle^{-1}$ (0.7–2.5 % $year^{-1}$). The genetic gains in IVDM were translated into greater animal production without decreasing the yield. Casler and Vogel (1999) reported that a 1 % increase in IVDM was correlated with 3.2 % gain in body mass of beef cattle. The IVDM of bromegrass was shown to increase by 9 g kg^{-1} while neutral detergents decreased by 8 g kg^{-1} (Casler et al. 2000).

Currently, forage breeding is facing many challenges due to rapid changes in land use patterns, i.e. conversion of grazing land to cropping land in traditional regions specialized in forage production (Argentina). The sustainable forage supply could be further affected by alternative exploitation of grass and pasture lands for

biofuel production. In view of the rapidly changing needs and demands of the livestock industry, forage breeding programs need to modify their goals and objectives accordingly. In the current scenario of continuous expansion of the livestock industry as a result of increasing human population, milk production is predicted to increase from 580 to 1,043 million mt, while meat production needs to increase from 210 to 527 million mt by the year 2050 to meet demand. Therefore, improving forage DM yield productivity and sustainability should be the major target of forage breeding programs. In this chapter we discuss current advances in forage crop ecology, and forage breeding objectives and procedures.

5.2 Forage Systems Worldwide

5.2.1 Grasslands

Grasslands are terrestrial ecosystems covered mainly by perennial herbaceous vegetation and usually dominated by grasses (*Poaceae*) (Tallowin and Jefferson 1999; Thomas 1980). Other vegetation complexes may also include legumes species (*Fabaceae*), sedges (*Carex*), rushes (*Juncaceae*) and herbaceous species belonging to many other plant families. Mediterranean semi-natural grasslands are dominated by annual species that are well adapted to the local highly variable climate (Cosentino et al. 2014). In the USA, grassland herbaceous plants other than grasses, sedges and rushes are commonly defined as forbs (Thomas 1980). In some types of grasslands (i.e. cerrado, desert steppes, shrubland) and under extensive land use systems (i.e. silvopastoralism, agroforestry) shrubs, trees or crops may also exist. The definition of grasslands has been extended by Peeters et al. (2014) to include *land devoted to the production of forage for harvest by grazing/browsing*, *cutting*, *or both*, *or used for other agricultural purposes such as renewable energy production*. Allen et al. (2011) emphasized that the term *grassland* is synonymous with *pastureland* and defined as *land* (*and the vegetation growing on it*) *devoted to the production of introduced or indigenous forage for harvest by grazing*, *cutting or both*.

Grassland ecosystems have evolved at many latitudes and climatic zones. Therefore, they encompass a wide range of habitats characterized by diverse agricultural management practices and socioeconomic and nature conservation values. The precise area of grasslands (defined as permanent meadows and pastures utilized for agricultural purposes) in the world is unknown, but it is estimated that grasslands occupy about 3.356 million ha, which corresponds to 68.6 % of the world's agricultural area (CSO 2012). In general, grassland area is commonly underestimated because of the lack of a uniform system of its categorization.

Grasslands can be categorized in many ways depending on the applied criterion. Grasslands that evolved natural plant communities are defined as natural or native

grasslands, while grasslands created as a result of human agricultural activity are categorized as agriculturally improved, cultivated grasslands or anthropogenic grasslands (Fritch et al. 2011). Peeters et al. (2014) introduced one more category of grasslands, e.g. grasslands no longer used for production. This category includes permanent grasslands which are maintained in good condition suitable for the production of bioenergy.

Natural or native grasslands are dominated by locally adapted, native plant species. Species composition and occurrence of these grasslands are unrelated to human activities. Native grasslands communities have evolved in areas where there are other natural ecosystems, i.e. forests or wetlands, could not thrive. Numerous climatic and environmental factors have contributed to the development of native grasslands, including low and high temperatures, strong winds, snow or stone avalanches in the mountains, short vegetation period associated with cool, rainy summer, low annual precipitation, high soil salinity, high level of water table and long periods of flooding in river valleys. Natural grassland ecosystems include steppes (also known as prairie in North America, pampas in South America, tussocks in New Zealand), savannas (Africa, South America, Australia), tundra (Alaska, northern Canada, northern Scandinavia, northern Siberia, Russia), shrublands, and temperate grasslands in the mountains (alpine grasslands) and in flooding areas in river valleys (Schultz 2005). Agriculturally-improved grasslands are subject to human activities like pasturing, mowing, use of commercial fertilizers or other agricultural treatments and require these management practices for their persistence. Managed grasslands dominated by indigenous or naturally occurring species are called semi-natural grasslands (Allen et al. 2011).

Grasslands can be divided into two main categories, i.e. permanent and temporary. Permanent grasslands are defined by Allen et al. (2011) as ecosystems with vegetation composed of perennial or self-seeding annual forage species which may persist indefinitely. The vegetation may include either naturalized or cultivated forages. Peteers et al. (2014) defined permanent grasslands as land used to grow grasses or other forages that has existed for 10 years or longer (this period may differ in some countries, but usually is not shorter than 5 years). Temporary grasslands are utilized for a short period of time, usually from one to a few years. Temporary grasslands are usually established on arable land and may be integrated into crop rotation (then the term *lay* is used). Pure stands of legumes or grass-legumes mixtures with a predominance of legumes are usually treated as temporary grasslands (Allen et al. 2011; Peteers et al. 2014).

Permanent and temporary grasslands may be grazed by animals (pastures) or may be mowed (meadows or hayfields). Allen et al. (2011) proposed to use the term *meadows* for natural or semi-natural grasslands only. Types of utilization (grazing, mowing) and its intensity (stocking rate, number of cuts during the vegetation period) are crucial for floral composition of grasslands, which in turn affects the sward height and its density, biomass production and the quality of forage.

5.2.2 Grassland Functions

Although the major role of grasslands is providing forage for livestock, they also provide a number of environmental services. The most important benefits from grasslands are:

(a) Furnishing habitats for wildlife, protecting and enhancing biodiversity. Grasslands constitute a vital part of the world's *biodiversity hotspots*, i.e. in the Mediterranean Basin over 50 % of the area is grown by grasslands and rangelands (Cosentino et al. 2014; Eurostat 2010).
(b) Protection and conservation of soil and water resources (preventing soil erosion on highly erodible lands, purification and filtration of surface water, reducing water runoff). The amount of eroded soil on slopes covered with perennial grassland vegetation is significantly less when compared with other cropping systems. For example, Cosentino et al. (2008, 2014) measured annual soil losses of 23 mt ha^{-1} $year^{-1}$ in annual tilled crops, while in crop rotations legume-cereal-brassica, the soil loss was only 15.5 mt ha^{-1} $year^{-1}$. The least amounts of eroded soil were measured in plots with alfalfa (*Medicago sativa* L.) (0.15 mt ha^{-1} $year^{-1}$), Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot] and tall fescue [*Schedonorus arundinaceus* (Schreb.) Dumort.] (1.8 mt ha^{-1} $year^{-1}$), subterranean clover (*Trifolium subterraneum* L.) (1.3 mt ha^{-1} $year^{-1}$), perennial *Miscanthus* sp. and *Medicago arborea* (0.1 mt ha^{-1} $year^{-1}$).
(c) Carbon sequestration in above and below ground plant biomass and in the soil contributing to reduction of the greenhouse effect at a global scale (Stypiński and Mastalerczuk 2006; Eriksen et al. 2012). Grasslands are able to sequester double the quantity of C in the soil in comparison to arable lands (Carlier et al. 2009).
(d) Source of renewable energy, i.e. an increasing percentage of grasslands is no longer used for livestock production in Central Europe but is used as a source of bioenergy (Rösch et al. 2009).

5.2.2.1 Biodiversity of Grasslands

Biological diversity, or biodiversity, encompasses the variety of life on earth. The term refers to each level of life organization, e.g. cellular level, species, and ecosystem (Fig. 5.1a). Genetic diversity refers to an array of phenotypic, biochemical and molecular characteristics within a species (Rauf et al. 2010). Genetic diversity occurs within and between populations of a species as well as between species. Species diversity refers to the number of species at a given time or in a specific ecosystem. Ecosystem diversity relates to the variety of habitats, biotic communities and ecological processes, as well as the diversity present within ecosystems in terms of habitat differences and the variety of ecological processes (CBD 1992; Swingland 2001). Biodiversity is not homogeneously distributed across the earth. Grasslands ecosystems are considered highly diverse, therefore it is critical to conserve biodiversity at the ecosystem level.

Fig. 5.1 (**a**) Grassland biodiversity, semi-natural *Molinietalia* meadow with *Dactylorhiza incarnata* (L.) Soó (*Orchidaceae*) at the Bug Ravine Landscape Park, Poland, (**b**) Evaluation of grass/legume mixtures at the experimental organic farm at Lindhof, Germany, (**c**) Evaluation study of teosinte (*Zea mays* ssp. *mexicana*) cv. PL-658198 at the College of Agriculture, University of Sargodha, Pakistan, (**d**) Evaluation plots of perennial ryegrass (*Lolium perenne* L.) and Italian ryegrass [*L. perenne* L. ssp. *multiflorum* (Lam.) Husnot] at the plant breeding company NPZ, Lembke, Germany (Photographs by: Imtiaz Akram Khan Niazi)

Despite the importance of natural and semi-natural grasslands, biodiversity resources within grasslands are declining (SCBD 2010). There are several reasons for the loss of grasslands biodiversity, i.e. management intensification (high fertilizers input, increasing stocking rates, increasing frequency of biomass removal, over-sowing, use of herbicides), abandonment, habitat fragmentation and isolation, conversion to arable land, afforestation, eutrophication (airborne nitrogen deposition), drainage of wetlands, urbanization (Steven and Lowrance 2011). Intensification of grassland management and grassland abandonment are considered especially

critical and have opposite effects on biodiversity. Under intensive management, biodiversity strongly decreases (Báldi et al. 2013), while abandoning agricultural practices results in a series of changes leading to a secondary succession (Batáry et al. 2011). The first trend can be illustrated with an example form Central Brazil where croplands and pastures have replaced nearly half of the cerrado ecosystem, a woodland savanna biome which is represented by a high number of endemic plant species. According to the SCBD (2010), the loss of the cerrado ecosystem was more than 14,000 km^2 per year during 2002–2008, or 0.7 % of its original area annually. This is a higher rate than the current rate of ecosystem loss in the Amazon. Secondary succession plays a significant role in the temperate European grasslands that are usually exposed to moderate disturbance events such as grazing, mowing or fires.

The occurrence of natural grasslands is predetermined by environmental conditions and wild herbivores. Semi-natural grassland ecosystems have been shaped during millennia by low-intensity, traditional management dated to the beginning of human agricultural activities during the Mesolithic-Neolithic transition. Depending on environmental conditions, grasslands may be climatogenic or anthropogenic. For example, in warmer and drier climates typical of steppes and savannas, the vegetation type is mainly predetermined by low precipitation, whereas the vegetation of alpine grasslands is determined by low temperature and short growing season. Semi-natural grasslands have persisted in environments that are less suitable for agriculture because of biotic and abiotic constraints. These grasslands are usually under some type of management, are not subject to heavy grazing pressure, frequent mowing, intensive fertilization, amelioration, soil cultivation, the use of herbicides, over-sowing or introduction of alien species. As low-intensity/low-output ecosystems, semi-natural grasslands are usually unprofitable. Semi-natural grasslands are very susceptible to secondary succession, thus they are considered one of the most endangered ecosystems in the world (SCBD 2010).

Preserving grassland biodiversity through extensive management systems is usually contrasted with requirements of intensive forage production (Isselstein and Kayser 2014; Nagy 2010), so the concept of grassland management practices integrating *sustainable development* with agriculture and conservation aims may be difficult to pursue. Biomass production from unfertilized, species-rich grasslands is less than that from improved, intensive grasslands based on a few highly productive grass and legume species and usually in a range of 20–80 % of the biomass produced by intensive grasslands (Tallowin and Jefferson 1999). Many countries allocate substantial resources to support the environmental function of grasslands, including biodiversity preservation and enhancement. For example, the European Union substitutes the loss of income for producers who decided to apply extensive grasslands management practices to protect the natural environment (Rehbinder 2013). Marquard et al. (2009) examined 198 grassland communities and found that biodiversity increased with plant density but it was independent of plant size. Plant density and size may be further affected by resource availability. Differences in plant density may also be driven by germination success, establishment and plant mortality. In contrast, Jamar et al. (2014) reported no relationships between herbage DM yield and the number of plant species in various grass-legume mixtures (2, 4, 8,

12 species) under contrasting management practices in a low-input system. The authors concluded that forage DM yield and its quality was primarily affected by defoliation frequency, the initial composition of species in mixed stands, and by growing season (Jamar et al. 2014).

The main anthropogenic factors affecting grassland ecosystems are fertilization and defoliation, either by mowing or grazing. Plant species-richness declines with increasing fertilizer inputs, especially nitrogen (Zechmeister et al. 2003) and phosphorus. Nitrogen inputs of 20–50 kg $Nha^{-1}year^{-1}$ result in 50 % reduction in the number of plant species (Plantureux et al. 2005). Phosphorus has been shown to have an even greater negative effect on biodiversity (Ceulemans et al. 2013). The effects of grazing and mowing on biodiversity depend on their frequency and timing. Usually, grazing by livestock is recommended to maintain biodiversity of grasslands (Bokdam and Gleichman 2000; Jerrentrup et al. 2014; Matera et al. 2010; Pykälä et al. 2005). Animals may influence the grassland vegetation by defoliation, seed dispersal, pulling up plants, supplying nutrients with excreta, or trampling. They can also affect spatial habitat heterogeneity by differentiation of the sward height (Durant et al. 2008; Jerrentrup et al. 2014). Middleton et al. (2006) indicated that mowing is an important management practice in wetland grasslands in Europe, applied as such for centuries. Kryszak and Kryszak (2006) examined the vegetation of excessively wet temperate grasslands in Poland and concluded that extensive management contributed to plant diversity and protected endangered plant and bird species inhabiting wetland meadows. Termination of grassland management always results in significant changes in the plant composition of anthropogenic grassland communities leading to a decline in their agricultural and ecological value. This is manifested by increasing encroachment of agriculturally undesirable plant species, shrubs and trees (Kryszak and Kryszak 2005) and inversely correlated with species richness and persistency of rare or endangered species typical for grassland ecosystems (Pykälä et al. 2005).

5.2.3 The Use of Legumes and Grass-Legume Mixtures

The presence of legumes in sward composition improves both soil properties and forage quality (Miller and Jastrow 1996; Sprent and Mannetje 1996). Cosentino et al. (2014) emphasized the role of annual and perennial legumes as components of native plant communities in fixing N_2, improving soil fertility by increasing organic matter content and improving physical and microbiological characteristics (Fig. 5.1b). In consequence, legumes are an important component of low-input production systems, mainly by reducing the need for inorganic fertilizers. Cosentino et al. (2003, 2014) reported that the amount of atmospheric nitrogen fixed by alfalfa, subterranean clover and *Vicia faba* ssp. *minor* is at 69–93 % of the total sward requirement, depending on the legume species and growing season. For example, the amount of fixed nitrogen in the first and the second year, respectively, varied

from 67–184 kg ha^{-1} in alfalfa to 136–162 kg ha^{-1} in *Sulla coronaria* L. Forage nutritional quality may be improved by adding legumes with greater contents of proteins, vitamins and minerals than grass species (Sturludóttir et al. 2014) which results in higher meat quality and animal average daily gain (Davies and Hopkins 1996; Maughan et al. 2014; Thomson et al. 1983). Legumes increase the content of linolenic and linoleic acids, and the proportion of unsaturated to saturated fatty acids in meat (Fraser et al. 2004). Some legume species, however, may contain anti-quality components (saponins, phyto-osestrogens, cyanogens, condensed tannins, quinolizidine alkaloids). Other legume species are particularly prone to infection with certain fungi during growth and hay storage. The fungal species are able to synthesize mycotoxins with detrimental effects on animal health (d'Mello and Macdonald 1996). The major disadvantage of legumes, however, is the ability to cause bloat in grazing cattle (Hancock et al. 2014). Individual legume species differ in their value as forage crops. For example, white clover often has poor persistence, low forage production in spring, and rapidly declining biomass production with stand age. Red clover and alfalfa are rather short-living crops, with adequate forage production during the first 3–4 years, are susceptible to pests, diseases and tramping. Overcoming these undesirable traits is a major challenge for forage legume breeders.

Mixtures of forage species belonging to different functional groups may be more advantageous than respective monocultures, in terms of higher biomass production and forage quality, decrease in seasonal variation in forage productivity, higher resource complementarity and reduced weed infestation (Finn et al. 2013; Hofer et al. 2014). Finn et al. (2013) evaluated 31 European grasslands ecosystems, including the Mediterranean Basin and found that mixed stands of four species yielded more biomass than any of the respective monocultures, irrespective of fertilization level. In Europe, the most common mixtures for intensively managed grasslands include perennial ryegrass, Italian ryegrass, *Festulolium* {a hybrid between perennial ryegrass and meadow fescue [*Schedonorus pratensis* (Huds.) P. Beauv.]}, tall fescue, meadow fescue, timothy grass (*Phleum pretense* L.), cocksfoot (*Dactylis glomerata* L.), white clover, red clover and alfalfa in different proportions depending on habitat conditions and type of land use.

5.2.4 Forage Germplasm Resources

The success of any breeding program depends on the availability of genetic variability within a species to achieve versatile breeding goals. Forage germplasm resources have been collected and preserved in various regions of the world. These efforts were centered at the Australian Tropical Forage Genetic Resource Center at Brisbane (ATCFC), Commonwealth Scientific and Industrial Research Organization (CSIRO) and the Centro Internacional de Agricultura Tropical (CIAT, Columbia), for the collection of tropical grass germplasm with the help of International Board for Plant Genetic Resource (IBPGR) along with national research institutes located

in Asia, Africa and the America. These accomplishments have contributed to the development of germplasm resource centers in the USA (Department of Agriculture), Brazil (Central Nacional de Recursos Geneticos e Biotechnologia) and Ethiopia (International Livestock Centre for Africa, Addis Ababa, ILCA). At CIAT, 23,140 specimens (127 genera and 700 species) of various species have been collected and preserved. These species include 21,460 legumes and 1,680 grasses from 72 countries. During 1993–1997, several expeditions were made resulting in the collection of almost half of the plant material preserved at CIAT. The germplasm was collected from Tropical America, Asia, Africa and parts of Oceania and Europe. ATCFC holds 39,000 plant accessions from more than 1,500 crop and forage species and their wild ancestors. The specimens stored at ILCA belong to 111 genera and 341 species. About 75 % of the genera are legume species, 10 % grasses, and 10 % shrubs and trees (browses). The collections include experimental lines and commercial cultivars. The commercial cultivars constitute 5 % of the total collections and are supplied to researchers for evaluation on a small scale. ILCA also has a partnership with CIAT for the introduction of tropical forages from South America. Species collected and stored at ILCA and its organizations include *Trifolium* sp., *Brachiaria decumbens*, *Panicummaximum*, *Macrotyloma* sp., lablab (*Lablab purpureus* L.), *Digitera* sp., *Pennisetum* sp., *Cynodon* sp. and *Chloris* sp. In China, 24,000 accessions were preserved and 15 nurseries of perennial grasses were established in various climatic zones of the country. The best known is a collection established in Ghuizhou province in the 1980s. This collection consists of 8,000 fodder accessions belonging to 1.410 species, 430 genera, and 86 families. About 800 of the accessions have been evaluated for forage production.

Seeds are stored in cold rooms (−20 °C) according to the recommended protocol adopted by IBPGR in 1985. The seed is stored after reducing the moisture contents to 3–7 % in dehumidifying room operating at 15 °C and 20 % relative humidity (Rao et al. 2006). Field gene banks have been established where large numbers of accessions have been planted to collect good quality seed produced under optimal conditions. Collected seed is then used to maintain the base collections and to supply seed for breeders. The seed is sealed in laminated aluminum bags. This method preserves seed viability for about 50 years.

Forage gene banks have increased in number from about 10 in the 1970s to more than 100 currently, mostly because of efforts of the germplasm resource centers. Sophisticated methods for seed collection, storage, cataloging and evaluating forage germplasm have been developed. Development of plant genetic resources has also contributed to the establishment of many basic principles of forage genetic resources, i.e. collection protocols and storage methods (Annicchiarico et al. 2015; Basigalup et al. 1995). Forage genetic resources in collections worldwide are much greater than their actual use in breeding programs. One disadvantage is that experimental data on forage germplasm are not widely available and future efforts of gene banks should include easier access to these data.

Germplasm resources are mainly utilized for cultivar development and improvement, and are comprised of wild populations, ecotypes and old cultivars. These germplasm resources are evaluated in various environments to identify the target

accessions to release as cultivars or utilization in breeding programs for incorporation of desirable genes in the cultivated germplasm. Germplasm is evaluated for morphological and biochemical traits to capture differences among accessions that could be utilized for development of cultivars with enhanced qualities. For example, such an approach is used in identification of sources for biotic and abiotic stress resistance to develop drought, salinity and disease resistant cultivars. Although there is broad diversity in modern forage species, a large number of ecotypes have not yet been tested for adaptation, agronomic and forage quality traits. Forage evaluation has been done in various parts of the world. In China, 14,757 accessions were evaluated for various agronomic traits related to forage yield and quality and 15,000 accessions were evaluated for biotic and abiotic resistance (Chen et al. 2009). In sub-Saharan Africa, forage legume resources were evaluated for forage yield and its component, forage quality and persistence (Thomas and Sumberg 1995).

In order to expand genetic diversity, germplasm with unique characteristics (traits contributing to high productivity and stress tolerance) has been introduced or exchanged, leading to the development of numerous landmark varieties (Thomas and Sumberg 1995). One example is the development of the tropical forage legume cultivar Stylo (*Stylosanthes guianensis* (Aubl.) Sw. var. *guianensis*) (Li et al. 2014; Phengsavanh and Frankow-Lindberg 2013). Stylo is more productive and has comparable forage quality with other legume cultivars, i.e. BRA 9690 (*Aeschynomene histrix*), and canavalia CIAT 17009 (*Canavalia brasiliensis*). The cultivar has been successfully acclimatized in various environments (Li et al. 2014; Varaporn et al. 2012).

5.3 Utilization of Forage Germplasm Resources

Forage germplasm resources may be evaluated for various economic traits and introduced as forage species or introgressed into elite germplasm to expand the genetic variances of cultivated germplasm. Many of the forage species have recently been domesticated (Miron et al. 2012). Compared to grain crops, domestication of forages has been more systematic (Lashley et al. 2014). As a result, forage species have significantly higher genetic diversity when compared with grain crops.

Wild species are often the source of useful alleles and may be used for the improvement of cultivated forage species, i.e. through development of interspecific hybrids. In maize, wild teosinte types (*Zea mays* ssp. *mexicana*) have been successfully used for the development of interspecific hybrids for high forage production and heat tolerance (Niazi et al. 2014, 2015). These wild teosinte types are also the source for resistance genes against pests and diseases (Niaizi et al. 2014), and to improve the methionine contents in forage and grain. Wild species of maize are known for their high protein content when compared with cultivated types and this trait may be transferred to cultivated germplasm (Niazi et al. 2015). In fact, intersubspecific hybrids between maize and teosinte have significantly higher protein content than both parents. *Sorghum bicolor* × *S. sudanense* hybrids have been developed to improve tolerance to frequent defoliation and to reduce HCN content in

Table 5.1 Successful examples of interspecific hybrids or their derivatives in achieving specific breeding objectives

Interspecific hybrids	Utilization	References
Zea mays × *Z. mays* ssp. *mexicana*	Hybrids with high forage yield, protein content, heat tolerance and stem borer resistance	Niazi et al. (2014, 2015)
Bracharia ruziziensis × *B. brizantha*	Development of spittle bug resistant cultivars for grazing	Felismino et al. (2012)
Lotus tenuis × *L. corniculatus* hybrids	Lower proanthocyanidins (PAs) in edible tissues to prevent bloating in ruminants	Escaray et al. (2014)
Trifolium repens × *T. uniflorum*	Backcross 1 showed 2–4 times higher shoot dry weight and resistance to drought	Nichols et al. (2014)
Pennisetum americanum × napiergrass (*P. purpreum* Schum.)	Hybrid Tift 23A × N23, yielded 34 and 27 % more dry matter	Hanna and Monson (1980)
Elephant grass (*P. purpureum* Schum.) × pearl millet (*P. glaucum*)	Hybrids combined resistance to drought, tolerance to diseases, and seed size of pearl millet with the hardness, aggressiveness, and high dry matter production of elephant grass	Obok et al. (2012) and Leão et al. (2011)
Buckwheat *Agopyrum esculentum* Moench.) × *Fagopyrum tataricum* (L.) Gaertn.	Trangressive segregants have improved self-pollination, fertility, frost tolerance, and higher content of beneficial compounds	Mendler-Drienyovszki et al. (2013)
T. pratense × *T. medium*	Selection was practiced for forage yield and persistence	Řepková et al. (2013)
T. uniflorum, *T. occidentale*, *T. pallescens*, 2x *T. ambiguum* and 6x *T. ambiguum*	Interspecific hybridization was done to transfer gene for the induction of tolerance to drought, diseases and salinity	Williams (2014)
Leucaena leucocephala × *L. pallida* (known as KX2)	Psyllid insect (*Heteropsylla cubana*) resistance	Dalzell et al. (2013)

leaves. Fang et al. (2012) noted that *Sorghum bicolor* × *S. sudanense* hybrids had HCN contents in the range of 5.80–10.43 mg kg^{-1} when plants were at 100 cm height, which was considered safe for grazing purposes. The DM yield and protein contents of these sorghum hybrids were superior to both parent species. Interspecific hybridization between elephant grass (*Pennisetum purpureum* Schum.) and pearl millet (*P. glaucum* L.) has been done to combine the resistance to drought, tolerance to diseases, and the seed size of pearl millet with the hardiness, aggressiveness and superior DM production of elephant grass (Leão et al. 2011). Wild sorghum germplasm has been a useful source for the new types of cytoplasm, cytoplasmic sterility, pest resistance, fertility restoration, hybrid vigor and apomixes. For example, wild pearl millet (*P. glaucum* ssp. *monodii*) germplasm has been the source of cytoplasmic traits for cultivar breeding (Hanna 1989; Rai 1995). Other successful examples of introgressions between wild species and their derivatives to achieve various breeding objectives are presented in Table 5.1.

Knowledge of genetic diversity and relationships among the germplasm accessions provides a useful tool for selection of parental lines to develop highly heterotic hybrids and transgressive segregants (Mohammadi and Prasanna 2003). Germplasm resources are evaluated on the basis of pedigree, phenotypic, biochemical and molecular diversity. Germplasm has been evaluated for molecular genetic diversity using marker such as ISSR (inter short simple repeat), SRAP (sequence related amplified polymorphism), RAPD (random amplified polymorphic DNA), RFLPs (restriction fragment length polymorphism) and SSR (simple sequence) (Wei 2004). The information generated has been helpful in parental selection to induce a higher percentage of heterosis and establishment of transgressive segregations. Molecular markers are being used to identify differences among genotypes, i.e. target alleles for the enhancement of forage related characteristics. Analysis of the studies shown in Table 5.2 indicates high genetic diversity within and between the ecotypes, suggesting that wild germplasm offers a great potential for selection and improvement of forage cultivars.

5.4 Forage Crop Phenotyping

Plant phenotyping is based on a broad set of techniques to obtain information regarding various traits, i.e. growth, biomass productivity, forage quality, persistence and resistance to biotic and abiotic stresses. In empirical plant breeding, traits related to plant architecture such as plant height, stem diameter, leaf area, tiller and leaf number, and canopy height are determined for the selection of appropriate genotypes. Growth-related traits such as leaf area expansion rate and biomass production are determined to measure the seasonal distribution and abundance of forage DM yield. Some physiological processes such as net photosynthesis rate, transpiration and stomatal conductance are used to determine the correlations with biomass production in a range of genotypes and environments (Huassain et al. 2015). Several of the physiological processes such as stomatal conductance and transpiration rate are particularly important under stress conditions and used as criteria for selection for sustainable yield. Biochemical traits such as chlorophyll, crude protein and fatty acid content are indirect measure of forage quality verified later in standardized laboratory tests of forage quality. Measurements of these traits by traditional procedures are often destructive, slow, expensive and cannot easily be applied to breeding populations. Technological improvements would greatly facilitate the capability for rapid phenotyping of the plants in the future. For example, devices used for measurement of total leaf area and many other traits have been improved to such an extent that over 1,000 plants may be phenotyped per day. Thermal techniques have been employed to measure canopy temperature through infrared thermometer. Canopy temperature depression (CTD) has been shown to be a highly integrated with root growth and transpiration and an effective technique to select for stress tolerant plants (Huassain et al. 2015).

Table 5.2 Genetic diversity of selected forage species

Species	Marker	Region	Ploidy	Genetic diversity	Reference
Buffalo grass *Buchloe dactyloides* (Nutt).	34 SRAP	North America	2×=20, 4×=40, 5×=50, 6×=60	0.33–0.99 HD=0.66	Budak et al. (2004)
				He=0.35	
Switch grass (*Panicum virgatum*)	85 RFLP	North America	2×=18	Higher in upland than lowland ectotypes	Missaoui et al. (2006)
	Loci		12×=108	Variation between ecotypes is higher than within ecotypes	
Pigeon pea (*Cajanus cajan*)	DArT	India, Africa	2×=22	Low genetic diversity among cultivars when compared with wild relatives	
Bermuda grass *Cynodactylon* spp.	15 AFLP), 10 (CpSSRLP), 10 (RAPD), (DAMD) primers	Turkey, USA	4×=36	A narrow genetic base; genetic similarity	Karaca et al. (2002)
			3×=27	(GS) with a range of 0.61–0.97	
Brachiaria ruziziensis	12 ISSR marker (inter-simple sequence repeat)	Brazil	2n=2×=18	Genetic similarity range 0.05–0.90	Azevedo et al. (2011) and Timbó et al. (2014)
			2n=3×=27		
			2n=4×=36	Higher genetic variation within populations than between populations	
Tall fescue	Diversity Arrays Technology (DArT)	USA	2n=6×=42	Narrow genetic diversity	Baird et al. (2012)
Sorghum *Sorghum bicolor*	40 EST-SSR	India	2n=2×=20	High genetic variance	Ramu et al. (2013)

Florescence meters measure chlorophyll content in a non-destructive manner and can be used to determine effects of a number of factors on plant growth. Particularly, the fluorescence chlorophyll meter is used in forage breeding to determine the stay-green trait under stress conditions and to determine the variability in the maturity trait among and within forage populations. Nuclear magnetic resonance spectroscopy (NMR) and near-infrared spectroscopy (NIR) are very time-saving methods to select for genotypes with high forage quality. The NIR method is much more convenient to determine crude protein, water soluble carbohydrates and lignin contents when compared with wet chemistry procedures. Plant imaging techniques, another example of non-destructive plant measurement techniques, have been successfully employed in forage plant breeding (Kalyar et al.

2013). Images are acquired automatically and stored in a database. Images are carefully evaluated to draw the desired information. Traits related to growth and forage productivity are determined from the image by calculation of shoot outline and pixel numbers within an image. The image background is precisely removed and other noises such as overlapping neighboring plants, dust contamination and insect damage can be corrected automatically through inbuilt imaging functions. Light intensity should be similar across all measurements and data points. Digital analysis of leaf area has been highly correlated with plant fresh and dry matter. Digital imaging analysis has also been widely used for plant height measurements to estimate correlations with fresh and dry matter. X-ray based computer tomography or a camera system inserted into soil has been invented to analyze root growth, total root length and root angle (Walter et al. 2012). The advanced root measuring techniques may help to select forage genotypes with highly efficient symbiotic relationships with the *Rhizobium* sp. bacteria and high nutrient use efficiency.

5.5 Metablomics in Forage Breeding and Genetics

Metablomics have been widely used to determine metabolites at the system level (Cao et al. 2012). Plant metabolites are the end products of cellular processes. Metablomics are powerful techniques for plant phenotyping and along with the genomics, proteomics and transcriptomics, are used to determine metabolite molecular pathways (Cao et al. 2012). Metabolites vary in size, structure and properties and affect key plant functions such as signaling, interaction with external environment, and resistance against biotic and abiotic stresses. The primary metabolites are directly involved in plant growth and reproduction. High resolution instruments such as mass spectrophotometer and chromatograph can identify thousands of peaks corresponding with a range of metabolites in a given biological sample. The high resolution peak quantification and reliable identification of metabolites require the optimization of peak retention time shift, variation of peak quantification between batches and run-order effects due to the decreased ionization (Cao et al. 2007; Chen et al. 2014). Metablomics are being used as a valuable tool for understanding the biochemical basis of phenotypic variation, i.e. to screen biologically important signals through computational and statistical tools (Broadhurst and Kell 2006). The information is then used to interpret the structure of the strong signals. The structural interpretations often identify the role of unknown or unexpected metabolites (Patti et al. 2012). In forage breeding, metablomics have been used to study the biochemical nature of polygenic traits and have increased understanding of the development of phenotypes for particular traits. The best known use of metabolomics is to determine biochemical basis for drought tolerance (Oliver et al. 2011; Sanchez et al. 2012). Knowledge of metabolites affecting forage quality traits such as lignin, cellulose or crude protein content has improved our understanding of breeding high quality forages and controlling cellular processes to decrease the conversion of complex 1 carbohydrates. With these techniques, genotypes could

also be discriminated for over-production of metabolites under stress conditions. In disease resistance breeding, defensive metabolites such as antibiotics and pigments may be used as selection criteria, instead of disease resistance scores. Metablomics could also be applied to genetic mapping to identify loci involved in the production of specific metabolites. Finally, disrupted metabolite production pathways have been exploited to understand the involvement of gene regulation and expression in forages (Rasmussen et al. 2012).

5.6 Important Breeding Objectives of Forage Species

The most important objectives of forage crop breeding involve improvement in the amount, quality and seasonal distribution of herbage yield, and tolerance to abiotic and biotic stresses. The objectives are particular to each species and climatic conditions (environmental adaptation) (Table 5.3). For example, major objectives of white clover breeding are high herbage yield and nitrogen fixation efficiency, competitive ability, tolerance to drought, winter hardiness, tolerance to grazing, resistance to pests and diseases and reduction in the potential to cause bloat in cattle (Rhodes and Ortega 1996). Often, an increase in plant persistence is achieved indirectly by breeding for resistance to diseases and pests (Rhodes and Ortega 1996). In the temperate regions of Europe, intergeneric hybrids of the *Lolium* and *Fesctuca* genera are of high interest (Thomas et al. 2003). These *Festulolium* hybrids have higher forage quality and are better adapted to a range of abiotic stresses than both parent species. *Lolium* and *Festuca* species offer valuable and complementary agronomic traits. Perennial ryegrass is characterized by good regrowth, high nutritive value and tolerance to grazing, while meadow fescue is more persistent and winter-hardy (Kopecký et al. 2006). Several amphidiploid cultivars of *Festulolium* have been developed. The major problem for amphidiploid breeding is a high level of homologous pairing between the parental genomes that leads to genetic instability and a loss of hybridity in later generations (Humphreys et al. 2014; Yamada et al. 2007).

In every climatic zone, specific factors determine the growth of plants. The most important factors include temperature and photoperiod that control phenology and play a vital role for plant adaptation. Temperature and photoperiod are determined by elevation and latitude. In the Nordic environments, the most limiting factors for growth of forage crops are a short and cool growing season and very low winter temperatures accompanied by prolonged snow cover, ice encasement, low light intensity, fungal infections under snow cover and occasional waterlogging (Helgadóttir et al. 2014). In contrast, in many warmer environments, i.e. in the Mediterranean, the major problem is drought stress during the summer period. Mediterranean grasslands consist mostly of annual species (Lelièvre et al. 2011). The herbage production period of annuals is short and it could be further compromised by the current climate change (Cosentino et al. 2014). Several cool-season perennial grass species with summer dormancy trait have evolved in association

Table 5.3 Major breeding objectives of forage crops

Crop species	Region	Breeding objectives	Major breeding procedures	References
Alfalfa	USA, Mexico, Argentina, Iran	Increase in crude protein digestibility and forage quality, tolerance to grazing, insects, diseases, salt, drought and heat stress and improvement in winter hardiness	Screening of elite and wild germplasm to introduce tolerance to biotic and abiotic stresses; development of di-haploid lines; induced polyploidy	Tucak et al. (2014) and Huyghe et al. (2014)
Sorghum	USA, Argentina, Mexico	Reduction of tannins and hydro-cynogenic glucoside; increase in seedling vigor and establishment; resistance to abiotic stresses, insects and diseases	Screening of elite and wild germplasm to introduce tolerance to biotic and abiotic stresses; induced mutations	Rao et al. (2013) and Haussmann et al. (2012)
Maize	USA, Russian Federation, Mexico, Canada, Eastern Europe	Dual purpose maize (forage and grain); introduction of stay-green trait; resistance to insects, borer and rodents, and to abiotic stresses; tolerance to frequent defoliation	Screening of elite and wild germplasm to introduce tolerance to biotic and abiotic stresses; recurrent selection; development of synthetic and hybrid varieties	Niazi et al. (2014, 2015)
Pearl millet	Russian Federation, China, India, Pakistan, Africa, USA	Heterosis, disease resistance to fungal diseases (smut and leaf blast), improvement in nutritional value at low soil fertility, increase in succulence, leaf thickness and water and soluble carbohydrate content	Hybrid seed development technology, cytoplasmic male sterile lines development, use of wild species for the introduction of novel traits, development of apomictic lines	Haussmann et al. (2012), Kountche et al. (2013), and Gupta et al. (2012)
Red clover	Argentina, Brazil, Sudan France, Romania, Croatia	Resistance to diseases, i.e. crown rot, root rot (*Fusarium* spp. and *Sclerotina* spp.) and insects, i.e. root borers, increase in soluble carbohydrates and DM digestibility, reduction in formonetin content, increased seed yield, fertility, seedling vigor, and establishment, improvement in the efficiency of symbiosis with *Rhizobium* sp. bacteria	Screening of elite and wild germplasm to introduce tolerance to biotic and abiotic stresses, development of di-haploid lines, induced polyploidy	Marshall et al. (2012) and Huyghe et al. (2014)

Berseem clover	Middle East, India, Pakistan, South Africa	Resistance to stem rot and root rot, increase in soluble carbohydrate content, reduction in protein degradation rate in the rumen, high tolerance to biotic and abiotic stresses, increase in nodulation capacity under alkalinity and salinity stress, decrease in self incompatibility, increase in seed size to improve seedling vigor, establishment and to increase fertility	Screening of elite and wild germplasm to introduce tolerance to biotic and abiotic stresses, development of di-haploid lines, induced polyploidy	Bakheit (2013) and Pecetti et al. (2012)
White clover	New Zealand, Australia	Phosphate uptake, pest tolerance, increase in adaptability and drought tolerance	Interspecific hybrids, marker assisted breeding	Huyghe et al. (2014), Forester et al. (2013), and Jahufer et al. (2013)

with annual components of Mediterranean grasslands (Naveh 1960). The use of summer-dormant perennial species could extend the grazing season, increase forage yield during winter growing season (Annicchiarico et al. 2013; Volaire 2008; Volaire et al. 2013). Adaptations of summer-dormant grasses to prolonged summer drought rely on an array of physiological and biochemical processes, including dehydration avoidance and tolerance mechanisms (Lelièvre et al. 2011; Malinowski et al. 2008a, b; Volaire 2008; Volaire and Lelievre 2001; Volaire et al. 2009). Recently, summer-dormant, cool-season perennial grasses have been used in place of traditional, summer-active cultivars in environments resembling the Mediterranean climate (Hopkins and Bhamidimarri 2009; Malinowski et al. 2009). Summer-dormant, cool-season perennial grasses can well tolerate repeated severe and prolonged summer droughts. Copani et al. (2012) denoted a relationship between summer dormancy level and the microclimate of the origin site in several populations of cocksfoot. They observed that the most of the summer-dormant populations evolved in environments with rainfall <600 mm year^{-1} and a dry period >120 days. Malinowski et al. (2008a) reported that summer-dormant types of cocksfoot and tall fescue expressed similar germination responses to photoperiod and the responses were different from their summer-active counterparts. This phenomenon was apparently associated with summer dormancy type and not with the origin of the accessions. The authors concluded that germination response to photoperiod could be used as a criterion to differentiate summer-dormant from summer-active types of perennial cool-season grasses.

In the past 10–15 years, several breeding programs of summer-dormant, cool-season perennial grasses have been established in Argentina (Amadeo and Guillén 2009), Australia (Culvenor 2009), France (Lelièvre and Volaire 2009), Italy (Pecetti et al. 2009), Morocco (Shaimi et al. 2009), and the USA (Hopkins and Bhamidimarri 2009; Malinowski et al. 2009). Summer dormancy is usually associated with low forage productivity of native summer-dormant cool-season grass populations (Shaimi et al. 2009); thus, forage breeders focus on the improvement of forage characteristics and seed yield in the three major grass species with the summer dormancy trait: tall fescue, cocksfoot and harding grass (*Phalaris aquatica* L.). In addition, breeders develop compatible legume companion species for the use in mixed stands with summer-dormant cool-season grasses in diverse environments, i.e. annual medics (*Medicago* sp.) and annual clovers (*Trifolium* sp.) (Butler and Malinowski 2012; Butler et al. 2011; Malinowski et al. 2008b). Recent climatic changes will impact many regions specialized in intensive livestock production (Lelièvre and Volaire 2009). Agronomists and plant breeders must find ways to adapt forage crops to these climatic changes by improving persistence, forage productivity and water use efficiency during the cool-season growth period. The summer dormancy trait may be the most viable option to match forage grass production with disturbed patterns of precipitation in the semiarid environments at latitudes 35–40° N and S, including California and the Southern Great Plains of the USA, Australia, Argentina and the Mediterranean Basin.

5.7 Breeding Objectives of Major Fodder Crop Species

5.7.1 Forage Yield

Forage yield is the amount of DM available from the pasture or rangeland on a hectare basis. Direct measurements of forage yield are not suitable selection criteria because of their destructive nature. Therefore, high-yielding forage genotypes in segregating populations are selected on the basis of secondary traits that are correlated with herbage production such as plant height, tiller number, leaf area, regrowth after clipping and plant maturity. Genetic gain depends on selection intensity, genetic variance, heritability of the secondary traits and correlations between secondary and primary traits (Price and Casler 2014a). Low broad-sense heritability was reported for dry matter yield (Ebrahimiyan et al. 2013; Price and Casler 2014b). Price and Casler (2014b) estimated moderate heritability for plant height (0.41) and high heritability for flowering date (0.75) in spaced-grown plants and concluded that these traits could be used as selection criteria for high forage productivity. Price and Casler (2014c) found that plant height, dry mass per plant and width of the second leaf were the best predictors of herbage yield in switchgrass.

Increase in winter rye biomass was the major breeding objective for the production of high forage and biofuel. Selection for biomass had moderate heritability (0.67–0.91) (Miedaner et al. 2010). There were positive correlations between biomass and early-season growth rate and heading date. Evaluation of teosinte germplasm showed the highest genotypic coefficient within germplasm for traits such as number of leaves per plant and leafiness ratio (Niazi unpublished data). Number of tillers of teosinte germplasm (Fig. 5.1c) also had a high genotypic coefficient. Broad sense heritability was high for all the traits except plant height, indicating that these traits could serve as tools for selection of high-yielding genotypes. Correlation analysis revealed positive and highly significant relationships between number of tillers and number of leaves per plant or number of leaves and leafiness (Niazi unpublished data). Positive correlation coefficient was also found between number of tillers and leafiness and between number of tillers and stem height. Positive correlation between the traits suggested that simultaneous selection of these traits could be made. Based on these results it can be concluded that number of leaves may be a good selection criterion for development of high-yielding maize genotypes as previously shown for teosinte (Bai and Rani 2000). The presence of a significant genotypic variation was noted for all measured traits. For example, teosinte accessions showed leaf numbers per plant of 25.33–260.47 and tiller numbers in the range of 1.17–13.00 (Fig. 5.2). Accessions PL-658198; PL-566685, PL-566682 and ACC-27460 had the highest number of leaves and tillers per plant (Fig. 5.2).

There are two approaches to improve the yield in forage crops, i.e. expand the yield potential of the forage or reduce the yield losses caused by various stresses. Selection for yield components could increase plant productivity but it may not

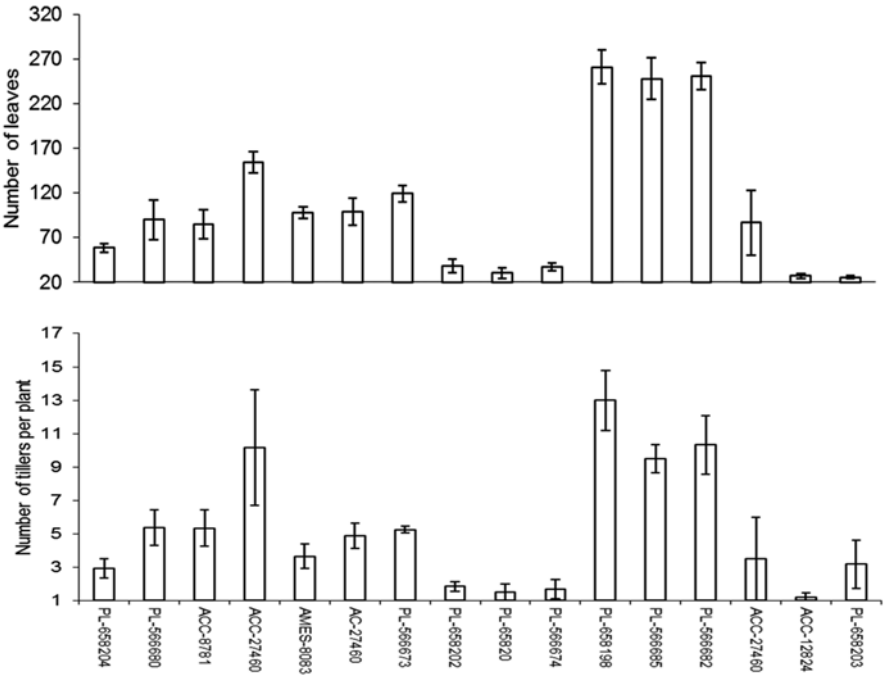

Fig. 5.2 Leaf number (*upper*) and tiller number (*lower*) per plant in experimental accessions of teosinte (Niazi, unpublished data). *Bars* indicate 1 standard error

capture tolerance to abiotic and biotic stresses. Improving forage yield potential along with traits inducing sustainability of yield under various environmental stresses is also important for forage breeders. Smith and Fennessy (2011) noted that improving drought tolerance through more extensive root system and higher growth rate after re-watering were important breeding criteria of perennial ryegrass under water-stressed conditions. For tall fescue, improvement of drought tolerance via selection for seedling vigor and persistence has been achieved.

Forage breeders are often confronted with the dilemma of selecting for desirable traits using spaced-grown plants or mixed populations (swards). Although swards provide a good estimate of forage DM yield, they are heterogeneous mixtures of various species and require a greater resource investment for evaluation. In contrast, spaced-grown plants allow for easy evaluation of a large number of genotypes with relatively lower efforts. As a compromise, forage breeders usually evaluate breeding populations as spaced-grown plants during early segregating cycles and select for traits such as disease resistance and forage quality. The improved selections are further evaluated and selected for forage DM production under sward conditions. Genetic gain for forage DM yield is successful under both selection systems. However, selection under sward condition may be more efficient and can reduce the number of breeding cycles.

5.7.2 Regrowth

The ability of forage plants to successfully recover from defoliation stress is the major key to maintain pasture and rangeland productivity. Forages with high regrowth ability are usually tolerant of grazing (Gao et al. 2008; Smith et al. 1989). Regrowth is initiated by three types of meristematic tissues (Chapman et al. 2012). In response to defoliation, regrowth is first induced in intercalary meristems below the leaf sheath, followed by the leaf primordial meristems. The axillary buds are the last to respond to defoliation stress. Intercalary meristem and leaf primordial meristem are active during tillering while axillary buds are active when defoliation occurs during internode elongation. The ability to regrow varies between species and depends on the height of defoliation (Shen et al. 2013). Under optimal fertilization and soil moisture, lower defoliation height may increase forage yield in alfalfa at the second harvest due to an increase in number of buds and shoots per plant (Shen et al. 2013). However, under stress condition, lower defoliation height decreased forage yield due to loss of apical meristems and energy storing tissues. Carbohydrates and nitrogen reserves also affect the regrowth (Klimeš and Klimešová 2002; Volenec et al. 1996). Lardner et al. (2003) compared regrowth rate of eight species in the pasture and noted that brome grass had the highest regrowth rate after defoliation, while timothy had the lowest regrowth rate. Some grasses induce regrowth through the nodes on the lower meristems of branches. This type of regrowth is called aerial branching (Van Minnebruggen et al. 2014b). Higher regrowth rate is correlated with greater persistence (Van Minnebruggen et al. 2014a, b). Robins et al. (2007) identified an MsaciB candidate gene for high forage yield and autumn regrowth in alfalfa. The ability of a genotype for bud formation after regrowth has been under the control of plant hormones such as strigolactone and auxins in red clover. The genotypes may be selected for the candidate genes (D27 and PNI) mediating the production of these hormones (Van Minnebruggen et al. 2014b).

Efficient regrowth is a major breeding objective in annual grasses. This trait is usually introduced from wild ancestors. For example, teosinte (the wild ancestor of maize) has higher regrowth rate and tillering than modern maize cultivars and can be harvested for forage multiple times during a growing season (Niazi et al. 2014). In sorghum, related species such as *Sorghum sudanense* and *S. halepense* have higher regrowth rate and tiller number, and provide 4–5 harvests per growing season when compared with cultivated sorghum. The use of wild ancestors was also successful in improvement of *Pennisetum* species, resulting in development of cultivars tolerant of repeated defoliation. In red clover, selection for higher number of first-order branches, short internodes and ability to resume growth after cutting increased the regrowth capacity of modern cultivars (Van Minnebruggen et al. 2014a).

5.7.3 Persistence

Persistence of forage plants is the ability to establish and maintain growth under prevailing environmental conditions (Marten et al. 1989). Plant persistence is a complex trait and is the sum of an array of other traits, including competitive ability and tolerance to abiotic and biotic stresses. Forage crops are exposed to a range of abiotic (moisture, temperature, soil mineral imbalance) and biotic (competition from other plant species, defoliation by grazing animals, harvest intensity and frequency) stresses (Bouton 2012a, b). Increasing plant persistence through induction of tolerance to abiotic stresses provides another option for the forage breeder to provide sustainability to the forage yield (Parsons et al. 2011). Inducing tolerance to abiotic stresses such as high temperature and drought may increase the survivability of forages and expand their availability (Huassain et al. 2015; Niazi et al. 2015). Drought resistance has been associated with persistence in a wide range of crops. In berseem, traits such as recovery rate after stress and canopy temperature depression index were useful in selection for drought resistance (Hussain et al. 2015). These traits were positively correlated with forage yield and ground cover under drought stress. In maize, the stay-green trait was transferred from the wild species into cultivated germplasm to increase plant persistence (Niazi et al. 2015). In pastures, forage plants are affected by a number of stresses imposed by grazing animals, i.e. specific biting patterns, selective removal of specific forage species or plant parts, saliva and urine residues and trampling. These biotic stresses may indirectly affect other forage yield limiting factors such as pathogens. Incorporation of traits inducing tolerance to grazing and trampling could help increase the persistence of forages. High grazing tolerance in forage crops depends also on other plant traits, i.e. the presence of a high number of stolons in alfalfa and white clover, creeping growth habit and reseeding ability (Bouton 2012b). Plant damage by insect, diseases and nematodes can further adversely affect the persistence of the forage crops. To increase plant persistence, forage breeding exploits natural variation in the germplasm such as modification in plant morphology and architecture, and the ability of plants to produce metabolites with insect deterrent or antibiotic properties (Niazi et al. 2014). In maize, however, several of the metabolites involved in insect deterrence may reduce forage palatability (Niazi et al. 2014).

Breeding for tolerance to abiotic and biotic stresses may affect forage quality. Some conventional breeding methods of inducing tolerance to environmental stresses (diseases, insects) are associated with a linkage drag, e.g. a phenomenon where undesirable genes are incorporated along with resistance genes into new germplasm. One approach to reduce the linkage drag is the use of molecular markers associated with undesirable genes.

5.7.4 Seed or Grain Yield

Most forages including grasses are poor seed producers (Vleugels et al. 2015). Low seed harvest index has been noted in various forage crops, i.e. 21–30 % in field pea (*Pisum sativum*), 21.2–31.0 % in oats and 12.6–22.2 in Italian ryegrass (Koc and Gul 2012; Siloriya et al. 2014; Simić et al. 2014). Seed setting is affected by both genetic and environmental factors, including temperature and photoperiod. High moisture during vegetative growth tends to delay the flowering and results in low seed yields (Fırıncıoğlu 2014). Genetic factors affecting seed production include self-incompatibility and high polyploidy levels that increase gamete sterility (Falcinelli 1999; Vleugels et al. 2015). Seed yield is highly affected by genotype × environment interactions (Bolaños-Aguilar et al. 2000, 2002). Selection for manual tripping in clovers has increased the self-fertility to 80 % (Abdalla and Zeinab 2012). Seed yield is often negatively correlated with total plant biomass. Cultivars with good persistence to grazing have usually low seed yields (Annicchiarico et al. 1999; Herrmann et al. 2006). Seed yield is also reversely correlated with lodging resistance and positively with harvest index (Koc and Gul 2012; Siloriya et al. 2014; Simić et al. 2014).

Seed weight per inflorescence has moderate broad sense heritability with very high genetic correlation with seed yield and harvest index. Other traits, including number of inflorescences and seed number per plant also showed significant phenotypic and genotypic correlations with seed yield. Head number per plant has been a successful trait for the selection of high-yielding genotypes in red clover (Herrmann et al. 2006). In field pea, the branch length had the highest direct effect on seed yield followed by 1,000-seed weight and number of seed pod^{-1} (Kosev and Mikic 2012). The stem biomass m^{-2} had the highest positive effect on seed yield in alfalfa (Iannucci et al. 2002). In forage crops, a significant variation for all seed yield components has been reported (Herrmann et al. 2006; Kosev and Mikic 2012).

Seed quality and seedling vigor are two of the most important traits in modern forage cultivars (Robins et al. 2012). Seedling vigor refers to a number of traits, i.e. high germination percentage, high shoot and root biomass and the ability for a rapid establishment. Seedling vigor may be improved by selecting genotypes for high individual seed weight. High seed weight is positively correlated with seedling emergence when planted at greater depth (Glewen and Vogel 1984) and reversely correlated with establishment time (Giordano et al. 2013). Seed size is highly heritable and can be used as a dependable selection criterion. The broad sense heritability of caryopsis weight in sand bluestem (*Andropogon hallii* Hack.) was 0.87 while narrow sense heritability for seedling weight, adjusted seedling weight, and forage DM yield were 0.37, 0.22 and 0.63, respectively (Glewen and Vogel 1984). About 50 % of the genetic variability in the seedling weight could be explained by variation in the caryopsis weight (Glewen and Vogel 1984). These results suggest that selection for seedling weight may bring genetic gains for caryopsis weight and seedling vigor. TeKrony and Egli (1991) reviewed relationships between seedling vigor and forage yield and noted that seedling vigor significantly affected and was positively correlated with forage yield harvested during vegetative phase or during

early reproductive phase. In grazing-type wheat (*Triticum aestivum* L.), large-seeded genotypes (≥0.24 cm seed length) had 13–26 % more ground cover than small-seeded genotypes (≤0.20 cm seed length) when sown at seeding rates based on an equal seed number (Bockus and Shroyer 1996). Large seeded genotypes produced 35–44 % more forage yield than small-seeded cultivars. In contrast, seed size had a marginal effect on growth and development of switchgrass (Smart and Moser 1999). In this grass species, seed size had no significant effect on shoot and root development, leaf area, shoot weight and production of adventitious roots at 10 weeks after emergence. Sidique (2015) studied correlation of various traits with seedling vigor in 29 berseem clover accessions. Result indicated that seedling vigor index was significantly and positively correlated with shoot length and seedling pulling strength. Furthermore, seedling pulling strength was positively correlated with root and shoot length.

5.7.5 Seed Production in Forages

Seed production is a multi-million dollar business across the globe. In 2012, import and export of field crop seed trade was over 5 million mt and worth about USD 13,015 million (International Seed Federation 2012). The USA is by far the largest forage seed producer, followed by the European Union, Canada, New Zealand and Argentina. The state of Oregon in the USA produces more forage seed than the rest of the world. Total grass and legume seed production in major countries was 910,000 mt in 2012 (International Seed Federation 2012). Among grasses, the most seed was produced for ryegrass (Fig. 5.1d) tall fescue and red fescue (*Festuca rubra* L.). Among forage legumes, the most seed was produced for alfalfa (47,416 mt), followed by common vetch, and red and white clover. European countries produce mostly forage grass seed. Significant production of forage legume seed (alfalfa, white clover) is conducted in France and Czech Republic (Huyghe et al. 2014). In Asian countries, there is a huge gap between the demand and supply of quality seed. For example, forage seed production in Pakistan is not more than 1 % of the total demand. In effect, only 7 % of the forage crop results from the use of quality seed. Consequently, forage production is based on expensive, imported high quality seed. For example, USD 6 million were spent by Pakistan for the import of berseem clover seed during 2014 (GOP 2014).

5.7.6 Classes of Seed

According to the official association of the seed certifying agencies, seed can be divided into four major classes:

Breeder Seed. The seed or vegetative propagated material which is under the direct control of the breeder or the developing institute. Breeder seed is the first genera-

tion seed of any breeder population, advanced lines, etc. It is the basic seed for the multiplication of foundation seed.

Foundation Seed. It is the progeny of breeder seed and is handled by a seed multiplication department. Foundation seed is multiplied under strict growth conditions approved by the seed certification agency. The seed is multiplied to maintain high genetic and physical purity of the cultivar. The seed is the source of all types of certified seed.

Registered Seed. It is the progeny of the foundation seed and is multiplied to maintain high genetic and physical purity. The seed is verified and approved by the certification authority for multiplication of certified seed.

Certified Seed. It is the direct progeny of registered or foundation seed. It is permitted to produce one or two progenies from the certified seed in case of highly self-pollinated crops. The seed is multiplied under strict growth conditions to maintain high genetic and physical purity. The seed is verified and approved by seed certification authority for sale to producers. Seed certification authorities inspect the crop and obtain seed samples to determine various seed standards. The seed is examined for off-types, weeds, diseases and insects presence. The seed sample is also examined for genetic and physical purity. Standards for genetic and physical seed purity as well as minimum germination have been developed by the Oregon Seed Certification Service for commercial forage crops (OCSH 2014).

5.7.7 Factor Affecting the Quality Seed Production

Several climatic factors affect seed quality and yield, i.e. temperature, humidity, photoperiod and soil moisture; thus, planting dates are important (Niazi et al. 2015). Seed production can be conducted in arid climates with supplemental irrigation. Legumes require ample moisture prior to blooming; thereafter the frequency and intensity of irrigation can be gradually reduced. Reduction in irrigation induces uniform blooming and reduces vegetative growth. High or very low moisture during the grain-filling stage may reduce the number of seed heads and seeds per head. High soil moisture induces vegetative growth at the expense of reproductive growth. In contrast, low soil moisture content results in shriveled seed with low germination rate. Legume crops such as alfalfa, red clover and berseem clover require bees for successful pollination and seed set. Therefore, bee colonies are often placed in the field to increase the chance for production of high seed yield. Other management practices include weed control and physical isolation of varieties to maintain seed purity and genetic purity. Low air humidity is required during seed maturation. Harvested seed is often dried to reduce moisture content and treated with fungicides to minimize losses during storage. Seed shattering at maturity, wet weather, insect damage during grain filling or drought stress may significantly reduce seed production of various forage crops.

5.7.8 Forage Quality

Forage quality is defined as the *extent to which forage can produce desired results in raising animals* (Casler 2001). Good quality forage should have high palatability, intake and digestibility, and be free from anti-nutritional compounds to assure high nutritional value. Nutritional value and digestibility of forage are determined by protein, water soluble carbohydrate, cellulose, hemicellulose and lignin contents. The presence of lignin and hemicellulose negatively affects forage quality and digestibility. Forage quality can vary widely with the type of plant tissues and their maturity. For example, delaying forage harvest of alfalfa by 1 week may result in a decrease in forage digestibility and protein content by 20 g kg^{-1} DM and an increase in cell wall concentration by 30 g kg^{-1} DM (Sanz-Sáez et al. 2012). Environmental factors, i.e. temperature and precipitation can affect forage quality by interacting with plant development and biochemistry (Azizi and Hajibabaei 2012; Niazi et al. 2015). Increasing forage digestibility is one of the most important objectives of forage crop breeding. Forage digestibility may be increased per se through selection for high levels of in vitro dry matter digestibility or indirectly through selection for high content of water soluble carbohydrates and proteins and low content of lignin and hemicellulose.

5.7.9 Cell Wall Digestibility

There is a significant variability in cell wall fractions of various plant tissues, resulting in variation in the energy value of the forage. In comparison with maize grain, the energy value of ryegrass forage is only 80 % and 33 % for wheat straw (Lundvall et al. 1994). Cell walls contain a high proportion of lignin which offers resistance to bacterial degradation, affecting forage digestibility and available energy to animals. In dicotyledonous species, the secondary cell wall of the xylem tissue of stem is the main compound affecting digestibility. Forage digestibility is measured as the percentage disappearance of plant compounds in the animal digestive tract. Improved digestibility of neutral detergent fiber of fodder grasses increases the forage intake and protein use efficiency by the ruminants (Jank et al. 2011). Forage species widely differ in digestibility. For example, perennial ryegrass has the highest and timothy the lowest cell wall digestibility among grass species. Tetraploid ryegrasses are superior to diploid types in terms of cell wall digestibility. Genetic variation for the trait is low within ryegrass families (Baert et al. 2014; Jung et al. 2012). Organic matter digestibility is correlated positively with neutral detergent fiber and negatively with total dry matter.

Improving cell wall digestibility is the main objective in forage plant breeding because it is correlated with the whole plant digestibility (0.6–0.9). Traditional plant breeding programs have only slightly improved cell wall digestibility. In some sorghum and maize cultivars, brown midrib trait was shown to reduce biosynthesis of

lignin in leaves (Jung et al. 2012; Lim and Taylor 2014). Transgenic techniques have been adapted to down regulate genes of the monolignol biosynthesis pathway (Lee et al. 2011; Zhao and Dixon 2011), but the effect was a reduction of plant agronomic fitness. Other approaches include breeding for reduced lignin/polysaccharide cross-linking, smaller lignin polymers, enhanced development of non-lignified tissues and targeting specific cell types to improve the cell wall digestibility (Jung et al. 2012; Voxeur et al. 2015). Brenner et al. (2010) identified polymorphism in O-methyltransferase genes that was associated with cell wall digestibility in European maize cultivars. Torres et al. (2014) showed a high degree of heritability ($\geq$0.65) for cell wall composition in doubled haploid population of maize. It was noted that a significant genotypic variation existed in the species for traits such as lignin, hemicelluloses and cell wall glucose release following sacharrification. Cell wall composition was shown to affect 52 quantitative trait loci (QTLs) distributed over 8 chromosomes.

5.7.10 Rumen Protein Degradability

Rapid degradation of proteins in forage by rumen microflora causes bloat in cattle and sheep. Protein degradation results in formation of foam in rumen which traps gases. This phenomenon is associated with forages high in protein content, i.e. clover and wheat. Reduction in protein degradability in the rumen is an important breeding objective in forages. This goal could be achieved by modifying the protein structure to reduce enzymatic degradation by rumen microflora and increasing digestibility of soluble carbohydrates. McRae et al. (1975) reported that freezing the forage could reduce protein degradability by 50 % in the rumen. Food supplementation with condensed tannins (CTs) is an effective way to reduce protein degradation in the rumen. The CTs bind with proteins under acidic to neutral conditions in the rumen, slow down the degradation rate and increase absorption of amino acids. It is estimated that CTs content of 2–4 % in forage DM can effectively reduce protein degradation (Kingston-Smith et al. 2013). Many forage species naturally contain CTs, i.e. bird-foot trefoil (*Lotus corniculatus* L.), but most of them do not, i.e. white clover, alfalfa (Forster et al. 2013). Legumes high in CTs content can be mixed with white clover forage low in CTs to reduce protein degradability. However, very high levels of CTs in forage may have an anti-microbial effect and reduce forage intake (McSweeney et al. 2001). Significant genetic variation in CTs content has been found within various species such as bird-foot trefoil, suggesting the potential to breed cultivars with recommended CTs concentrations (Marshall et al. 2008). Breeding approaches such as isolation and transformation of genes related with the CTs biosynthesis pathway have also been proposed (Dixon et al. 2013). Genes such as TrCHSh were isolated from the stolon tip of bird-foot trefoil and TrBANa, TrLARb were isolated from the inflorescence (Panter et al. 2005). These genes have been used to produce transgenic white clover plants with increased CTs content (Kingston-Smith et al. 2013).

5.7.11 Increasing Forage Conversion Efficiency in Rumen

Ruminants produce nitrogen and methane as a byproduct of their metabolism (Cottle et al. 2011; Doreau et al. 2014). About 37 % of the global methane production comes from the microbial fermentation of lignin-cellulosic compounds in the ruminant digestive tract (Cottle et al. 2011; Grainger and Beauchemin 2011). The efficiency of animals to convert the feed nitrogen compounds is poor. About 70 % of the nitrogen is returned to the ecosystem and serves as a substrate for the production of nitrous oxide through microbial processes (Hristov et al. 2013; Peters et al. 2013). Nitrous oxide is one of the greenhouse gases contributing to raising global temperatures. Improving the forage conversion efficiency (ratio of final product to the metabolized energy) in the rumen would help to improve animal gain and reduce the production of environmental pollutants such as nitrous oxide. The relative feed energy is considered a major predictor of its digestibility (Basarab et al. 2013; Waghorn and Hegarty 2011). Feed energy conversion could be improved by increasing forage DM digestibility, absorption efficiency of amino acids in the rumen, the proportion of water soluble carbohydrates and proteins, and reducing the proportion of lignin and cellulose. A slower digestion rate of feed proteins and improved balance through temporal separation of nitrogen and energy sources availability can also increase the efficiency of forage digestion in the rumen. Surplus energy sources (simple carbohydrates) should be available during earlier stages of the fermentation process to provide an alternative to the readily available nitrogen in the forage (Basarab et al. 2013), i.e. by increasing the proportion of water soluble carbohydrates instead of complex carbohydrates (lignin, cellulose). Genotype selection for increasing water soluble carbohydrate content has been successful in perennial ryegrass, resulting in higher milk production by cows and meat production by sheep (Lee et al. 2001; Miller et al. 2001). Perennial ryegrass has been transformed using the fructosyl transferase gene for over expression of the fructan production. The transformation improved the available energy by one MJ of metabolized energy per kg of DM forage (Spangenberg et al. 2011, 2012).

5.7.12 Increasing Nitrogen Fixation Efficiency in Forage Legumes

Nitrogen fixation by legume species represents the major source of nitrogen for both plant and soil, reducing the input of commercial N fertilizers and providing sustainability to the ecosystems. Improving the nitrogen fixation efficiency by forage legumes is an important objective for breeding programs worldwide. Legume species vary in their ability to fix atmospheric nitrogen that can reach from 10 to 250 kg N ha^{-1} $year^{-1}$ (Roscher et al. 2011). The efficiency in fixing atmospheric nitrogen depends on the legume species, rhizobia strains and environmental factors (Peoples et al. 2013). Among legume species, the highest nitrogen fixation capacity was

noted for white clover (545 kg N ha^{-1} $year^{-1}$), followed by the red clover (373 kg N ha^{-1} $year^{-1}$) and alfalfa (350 kg N ha^{-1} $year^{-1}$) (Carlsson et al. 2003). Broad genotypic differences in the ability to fix nitrogen were also reported for other species such as common bean (*Phaseolus vulgaris* L.), field pea and lentil (*Lens culinaris* L.) under controlled growth conditions (Abi-Ghanem et al. 2011, 2013; Devi et al. 2013). In general, environmental conditions that favor growth of legume host species and survival of rhizobia increase the atmospheric N_2 fixation.

Biotic and abiotic stresses are major constraints in full expression of the genetic potential of host and rhizobia species to fix nitrogen (Miklas et al. 2006). Thus, effectiveness of nitrogen fixation can be improved by development of legume cultivars tolerant to these stresses. Soil microbiologists have identified *Rhizobium* sp. strains with higher nitrogen fixing efficiency. The ability to fix nitrogen by the rhizobia strains was positively correlated with aboveground biomass of legumes (Hardarson 1993). The genotypic differences are based on allelic variation in the accessions that allow for a better compatibility with rhizobia strains. Numerous methods have been employed to measure nitrogen fixation under field conditions (Wilson et al. 2012). The natural abundance of the δ15N isotope is the most common method for determination of nitrogen fixation in legume species (Carlsson and Huss-Danell 2003). Under controlled conditions, nitrogen fixation efficiency may be measured by the activity of nitrogenase using acetylene reduction assay (Mundy et al. 1988). Phenotypic evaluation of the germplasm for nitrogen fixation efficiency may be indirectly measured by the frequency of nodule formation, nodule weight, root weight, total plant nitrogen and residual nitrogen content in the soil (Abi-Ghanem et al. 2013). The ability to fix nitrogen has moderate heritability and could be improved through selection in transgressively segregating generations for positive alleles regulating nitrogen fixation (Hwang et al. 2014; Inuwa et al. 2012). Genotypes with high *Rhizobium* sp. infection rates could be used as a selection criterion for increasing the efficiency of nitrogen fixation in legumes (Abi-Ghanem et al. 2011).

Molecular tools such as retrotransposons have been successfully used to tag genes regulating nitrogen fixation in host plants, i.e. *Medicago tranculata* (Pislariu et al. 2012). After insertion of the mutations, populations were characterized for their ability to produce nodules and categorized as non-nodulating mutants, non-functional nodulating mutants, partially functional nodulating mutants and mutants with the abnormal nodule emergences, elongation and nitrogen fixation. These mutants served as populations to identify the genes related to nodulation. In alfalfa, down regulating the genes mediating monolignol biosynthesis enzyme, e.g. hydroxycinnamoyl coenzyme A – shikimate hydroxycinnamoyl transferase, responsible for lignin formation has increased the efficiency for nodule formation (Gallego-Giraldo et al. 2014). Higher nodule formation efficiency was associated with greater concentrations of gibberellins and flavonoids in roots. Nitrogen fixation efficiency by forage legumes can also be affected by pasture management practices and soil composition and texture. Therefore, the environmental impact on nodule formation and nitrogen fixation efficiency in forage legumes needs to be considered in breeding strategies (Unkovich 2012).

5.8 Breeding Procedures

5.8.1 Half-Sib Mating

Economic traits such as forage yield and quality, and plant persistence are quantitative in nature and are significantly affected by the environment (Faville et al. 2012; Serba et al. 2015). Therefore, it is important to determine the magnitude of genetic variation, or proportion of genetic variation from total variation, associated with particular traits. Knowledge of the type of genetic variation is necessary for adoption of appropriate selection procedures. Depending on the complexity of intra- or inter-allelic interactions, three types of genetic variance are recognized, e.g. additive, dominance and epistatic (Bhandari et al. 2011; Mushtaq and Gull 2013). A majority of the selection procedures use additive variance; thus, the proportion of additive variance to the total phenotypic variance could indicate the genetic gain resulting from the selection. If the additive variance is high, a phenotypic selection is an appropriate approach. The mode of gene expression would also affect the traits a cultivar is bred for (Bhandari et al. 2011). Synthetic or open populations are often developed as a result of recurrent selection based on additive variance. Hybrid varieties are characterized by the presence of gene over-dominance or epistatic interactions (Niazi et al. 2015). In order to gain information about the type of gene interactions, heritability, genotypic correlations and prediction of genetic advances, half-sib families are developed through various mating designs, i.e. diallel, nested and factorial designs (Nguyen and Sleper 1983). These designs differ from each other in the degree of homozygosity of the parents and the mode of pollination. In diallel advanced generations, parents with a high degree of homozygosity are mated in all possible combinations, allowing for investigation of their combining ability (Nguyen and Sleper 1983). Factorial or nested designs are often used to determine the genetic variance associated with desirable traits.

In forage crop breeding, the techniques of polycross and open-pollinated mating are used to generate half-sib families through random pollination among the parents. In this process, a large number of combinations can be obtained provided the parental plants have synchronous flowering, there is a sufficient replication of clones and they genotypes are compatible. In polycross nurseries, random mating is ensured by providing equal chance to all parents to pollinate each other. This often requires a higher number of replications (9–10), randomization and isolation. Half-sib families may also be developed using the top-cross method (Casler and Charles 2008). In this method, each line is crossed to a common tester. The tester could be an open-pollinated population with a broad genetic base. Analysis of variance on half-sib families and parental clones, and analysis of covariance between the parents and offspring provides information about additive variance associated with desirable traits. Cumulated additive variance is used to estimate narrow-sense heritability. Narrow-sense heritability is an index of selectable variation associated with particular traits (Nguyen and Sleper 1983). Narrow-sense heritability can be estimated by doubling the linear regression coefficient between the half-sib progenies

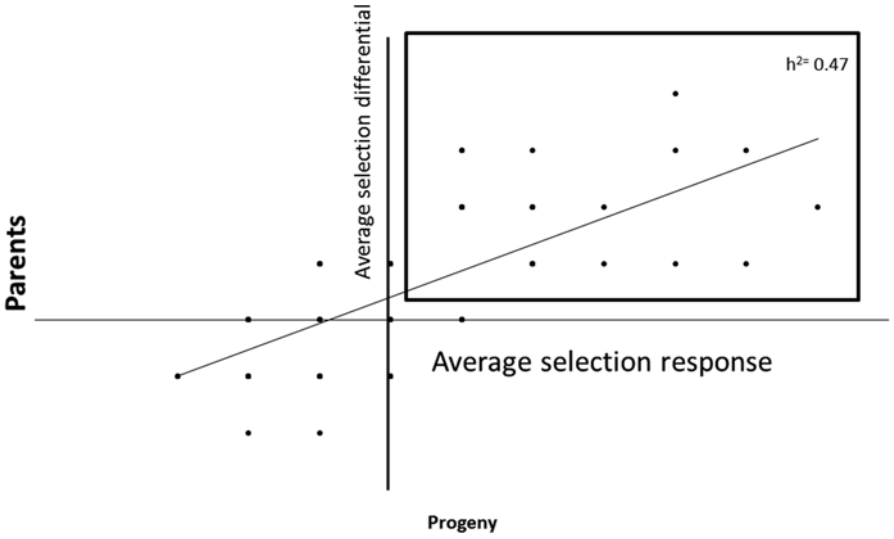

Fig. 5.3 Selection of parents and progenies for tillering capacity in the F_3 population of maize × teosinte in 2013 (Niazi unpublished data)

and their parents. A good selection response for the tillering ability in maize was noted which was indicated by the high narrow sense heritability ($h^2 = 0.47$) when maize progenies were regressed against the parents (Fig. 5.3).

5.8.2 Recurrent Selection

In forage breeding programs, recurrent selection is utilized to systematically increase the desirable alleles in the population (Hallauer and Darrah 1985). A rapid fixation of alleles through self-pollination decreases the chance for selecting an appropriate genotype with desirable alleles (traits) (Resende et al. 2013). For example, the chance for the fixation of five favorable alleles in a single genotype through self-pollination is 1/32 while through recurrent selection it increases to 1/13. Recurrent selection, however, is not a common breeding method in forage crop species, likely because of the slow progress in genetic gain, the requirement for several selection cycles and the difficulty in applying it in self-pollinating populations (Annicchiarico et al. 2015). The immediate outcome of the recurrent selection is an open-pollinated population which can be converted into a synthetic population. Nevertheless, there are examples of forage crop cultivars developed through recurrent selection (Burton 1982; Casler 1999; Jacob et al. 2014).

Selection gain is determined by the three factors, e.g. selection differential, narrow sense heritability associated with traits of interest and parental control (C) (Hallauer and Darrah 1985). If selected plants are recombined, then parental control

Table 5.4 Correlated response to the selection for multi-tillering in maize × teosinte hybrids

Source	Plant height	Forage DM yield	Leafiness	Leaf number plant^{-1}	IVDM
Un-selected F_3 population	84.39 ± 27.58	354.63 ± 31.57	0.26 ± 0.14	21.07 ± 5.38	61.04 ± 11.29
Selected progenies	69.74 ± 19.67	337.58 ± 18.54	0.47 ± 0.10	32.17 ± 3.91	69.58 ± 5.22

is C = 1/2 because the recombined population will only possess desirable recessive alleles from selected maternal plants and not from the random paternal plants. In contrast, when parents of superior half-sib progenies are used to create a recombination block, parental control C = 1 because there is control over both parents and it is likely that the progeny will receive desirable alleles equally from both parents. Recurrent selection can be applied at the phenotype or genotype level. Phenotypic recurrent selection is done on the parental plants while genotypic recurrent selection is done on the progeny population. Although phenotypic recurrent selection may result in forage DM yield improvement, this method is not widely used because of marginal or no genetic gains (Resende et al. 2013). To improve the forage DM yield of maize, Niazi et al. (2014) applied selection for tillering in the F_3 population and selected 84 plants with multiple tillers. These selected plants were allowed to cross pollinate. Selection differential was 5.0 tiller plant^{-1}. The progenies showed selection response of three tillers plant^{-1} and heritability of 0.47. Progenies with higher tiller numbers than the average tiller number measured for the parental plants were used in the next cycle of genotypic selection. Selection for multi-tillering showed correlated response with selected traits determining forage DM yield (Table 5.4). There was no significant effect of the recurring phenotypic selection on plant height and forage DM yield. In contrast, selection for multi-tillering significantly increased leafiness and number of leaves per plant which also increased forage quality, succulence and intake by animals (Niazi et al. 2014).

5.8.3 Hybrid Breeding

Hybrids are produced from controlled mating of two parent components, i.e. an ith line mated with a jth line. The seed obtained from this cross is called as F_o and is used to produce a F_1 population also defined as hybrid population. Hybrid populations exploit both general combining abilities (GCA) and specific combining abilities (SCA) (Singh et al. 2014). Hybrid populations are superior in forage yield, quality and resistance to stresses when compared with open-pollinated or synthetic populations. Hybrid performance depends on the magnitude of heterosis, defined as superior performance of the F_1 population over the median value [(ith + jth)/2] for any desirable trait in the parental population. The magnitude of heterosis is determined by the genetic distance, directional dominance and compatibility of the parental lines (Niazi et al. 2015). Genetic distance between the parental lines may

be estimated by phenotypic and molecular markers (Rauf et al. 2010). Despite a large number of experimental forage hybrids developed every year, only few are commercialized. Factors affecting development of hybrids include the presence of male sterility in female lines, synchronized flowering and high fertility of parental lines (Brummer 1999; Cai et al. 2013; Kobabe 1983).

5.8.4 Type of Forage Hybrids

There are several types of hybrids, depending on the degree of heterozygosity and the number of inbred lines used for their development (Brummer 1999).

Population/Semi-hybrids

$$\text{Population A} \times \text{Population B}$$

Single Cross

"A" (cytoplasmic male sterile) × "R" (restorer line)
"A" (photo-thermo sensitive male sterile; male fertile dominant female but self-incompatible) × male (cross-compatible with the female line)

Double cross

$$(i \times j) \times (k \times l)$$

where i (male sterile), j (non-restorer to fertility), k (male sterile) and l (restorer to fertility) are inbred lines.

Hybrids differ in their heterogeneity. Population hybrids are heterogenous and heterozygous, while single-cross hybrids are homogenous and heterozygous (Posselt 2010). Semi-hybrids have a broad genetic base and phenotypic flexibility to adapt in variable environmental conditions. Single cross hybrids are heterozygous and more uniform than double-cross and semi-hybrids for traits determining forage productivity. Semi-hybrids have been developed in numerous forage species to avail natural heterosis (Riday et al. 2002). Population and single-cross hybrids differ in the expression and type of heterosis. Heterosis in single-cross hybrids is based on inbred mid-parent heterosis (IPMH), while panmictic mid-parent heterosis (PMPH) is estimated in population heterosis.

5.8.5 Development of Maize × Teosinte Hybrids for High Forage Yield

Forage DM yield of maize cultivars can be improved by hybridizing them with wild maize ancestors, namely teosinte. Inbred maize lines were developed by self-pollinating selected plants in an open-pollinated population (Niazi unpub data).

Table 5.5 Mean genetic variability of selected growth parameters in parental and hybrid populations of maize and teosinte

Source	Plant height (cm)	Leaf weight (g)	Stem weight (g)	Tillers plant^{-1}	Number of grains cob^{-1}
Teosinte (T)	78.5 ± 20.1	72.3 ± 8.1	216.3 ± 14.3	1.85 ± 0.61	–
Maize (M)	123.4 ± 12.2	47.9 ± 5.6	147.5 ± 8.3	1.00 ± 0.00	630.5 ± 20.1
M × T (F_1)	169.9 ± 15.7	91.7 ± 6.2	260.5 ± 8.6	1.40 ± 0.64	257.3 ± 40.1
$F_1 \times F_1$ (RMP)	164.2 ± 10.7	82.2 ± 3.1	234.2 ± 9.2	1.75 ± 0.51	151.3 ± 21.6
M × T ($F_{2'}$)	145.2 ± 33.9	75.4 ± 4.2	256.8 ± 13.3	1.37 ± 0.61	115.2 ± 52.2
σ^2Genotypic	502.2	611.2	1085.3	0.09	–
σ^2Environment	686.4	311.6	5312.2	0.23	–
σ^2Phenotypic	1188.6	923.2	6397.1	0.32	–
Heritability	0.42	0.66	0.83	0.28	–

Source: Niazi et al. (2014)

Five maize inbred lines with good combining ability were then selected and crossed with 16 teosinte lines to develop 80 inter-subspecific hybrids (Niazi et al. 2014). These hybrids, planted on two sowing dates, expressed superior forage DM yields and other traits related to forage quality. Forage DM yields of the hybrids depended on the specific combining ability and the genetic distance between the parents calculated on the basis of various morphological traits (Niazi et al. 2015). Inter-subspecific hybrids were taller than their parents, had greater leaf area expansion and number of tillers, regardless of heat stress. Inter-subspecific hybrids expressed 68 %, 53 % and 43 % inbred mid-parent heterosis for forage DM yield, leaf and stem biomass, respectively (Table 5.5). Promising F_1 genotypes were intercrossed and their progenies were evaluated for traits determining forage DM yield. PMIH was 62 %, 37 % and 29 % for DM yield, leaf, stem biomass and number of tiller plant^{-1}, respectively.

5.8.6 Polyploidy

Polyploidy is a widespread phenomenon among forage crops. Many forage crops such as ryegrass (2×, 4×), alfalfa (4×), white clover (4×) and berseem clover (4×) are natural auto-poplyloids. Polyploidy offers certain advantages such as asexual mode of propagation, high foliage biomass, higher resistance to abiotic stresses, higher persistence or higher regrowth rate after defoliation (Kopecký and Studer 2014). At the cellular level, neopolyploid cells have better fitness and adaptability to stresses than haploid cells because of higher volume to surface ratios. The high volume of polyploid cells may also increase fresh biomass and palatability of forage (Niazi et al. 2014). Niazi et al. (2014) noted that induced polyloids of maize and inter-subspecific hybrids had greater leaf area per plant and over-expression of leaf soluble proteins and crude protein than the diploids. Induced polyploidy could also

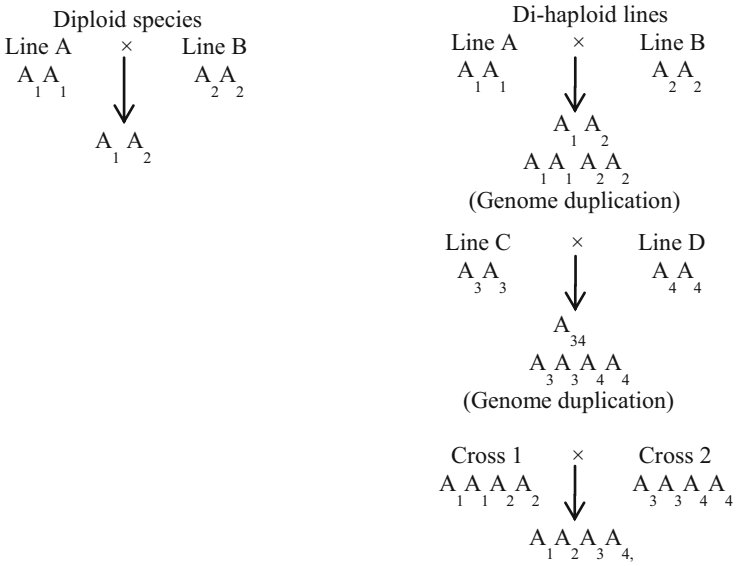

Fig. 5.4 Theoretical maximization of hetrozygosity in diploid and auto-tetraploid species

reduce the heterosis decay in the auto-tetraploid maize lines. Genetically, auto-polyploid plants are the source of new alleles at specific loci (Acuña et al. 2011). A diploid species carries two copies of alleles in a homozygous or heterozygous form, while an auto-tetraploid species has four alleles at specific loci. Therefore, auto-tetraploids are more likely to undergo mutations of alleles at specific loci. In the presence of a mutagen such as ethyl methane sulfonate, auto-polyploid cells have shown better fitness than haploid cells for many generations due to masking of deleterious alleles in polyploids. In diploids, two contrasting alleles are sufficient to maximize the heterozygosity at specific loci while in auto-tetraploids four alleles are needed. Therefore, breeding of auto-tetraploid species is more complex than diploid species. A theoretical example of the maximization of alleles in diploid and tetraploid species is presented in Fig. 5.4 (Riddle and Birchler 2008). Based on the proposed model, double crosses and single crosses in auto-tetraploid species were compared (Dunbier and Bingham 1975; Groose et al. 1989; Niazi 2014; Sockness and Dudley 1989). Superiority of double-cross induced tetraploid hybrids of maize resulted from four different alleles at one locus (Riddle and Birchler 2008). In natural auto-tetraploids of alfalfa, a heterosis increase of 114 and 125 % was reported for three-way and double-cross hybrids when compared with single-cross hybrids. Dunbier and Binghma (1975) noted superiority of double-cross over the single-cross hybrids for forage DM yield, reproductive fertility and seed weight of alfalfa.

Niazi et al. (2014) created hybrids between teosinte and maize that were subsequently subjected to colchicine treatment for the induction of polyploidy. Induced autotetraploid, inter-subspecific hybrids were superior to diploid hybrids in traits such as leaf biomass and leaf area, but not for stem biomass or plant height.

Table 5.6 Mean comparison of single and double cross hybrids at diploid and tetraploid levels for plant height (*PH*), leaf area (*LA*), leaf biomass (*LM*), stem biomass (*SM*) and crude protein content (*CP*) (Niazi 2014)

Intersubspecific hybrids	PH	LA	LM	SM	CP
Maize × teosinte (F_1,2×)	171.43 ± 5.16	544.29 ± 32.27	93.35 ± 14.24	216.38 ± 8.89	14.29 ± 0.89
Maize × teosinte (C3,4×)	144.29 ± 9.34	664.27 ± 21.34	112.29 ± 8.37	189.34 ± 9.64	15.11 ± 1.34
$F_1 \times F_1$ (2×)	159.34 ± 8.27	527.36 ± 16.33	81.27 ± 8.35	202.48 ± 8.65	13.93 ± 0.87
$F_1 \times F_1$ (4×)	189.38 ± 10.31	714.35 ± 18.65	126.71 ± 6.67	234.58 ± 7.34	15.57 ± 0.88

C3 is a colchiploid selfed population grown for two generations. Mean values for diploid intersubspecific hybrids were averaged over 16 hybrids, while colchiploid hybrids were averaged over 9 hybrids. Mean values for double crosses were averaged over 4 hybrids

Promising auto-tetraploid single crosses were selected and intercrossed to develop double-cross hybrids. Double-cross hybrids were compared with single-cross hybrids at diploid and auto-tetraploid levels for traits related to forage yield and quality (Table 5.6). Results suggest superiority of auto-tetraploid double-cross hybrids for all traits related to forage yield, except for leaf area and leaf biomass. Colchiploids (4×) of double-cross hybrids showed superior performance for leaf area and leaf biomass when compared with diploids (2×) and induced tetraploid (4×) hybrids of single crosses.

5.8.7 Di-haploid Breeding in Forages

In auto-tetraploid forage cultivars, di-haploid (2×) breeding is practiced to isolate advanced breeding lines to increase the heterosis, QTL mapping or establishment of new segregating populations. The advantages of di-haploid breeding in auto-tetraploid species include simple inheritance and therefore, selection could be more effective at the diploid level (Groose et al. 1988). Greater selection gains can be obtained through gamete selection (Niroula and Bimb 2009). The di-haploid lines could be used in crossing schemes both at a diploid (2×) or a tetraploid (4×) level. The di-haploid lines may be directly combined in various mating designs to estimate general and specific combining ability. Isolated lines may be subsequently doubled to induce tetrasomic homozygosity. The di-haploid lines (2×) are relatively less fertile as compared with tetraploid lines (Groose et al. 1988). Therefore, di-haploid lines are often used as female parents to generate F_1 crosses. There is no correlation between the forage yields of iso-genic diploid hybrids when compared with tetraploid hybrids of alfalfa (Bingham et al. 1994). Isogenic tetraploid crosses were 25 % more heterozygous at RFLP loci than the diploid lines of alfalfa species (Kidwell et al. 1994). The performance of tetraploid hybrids of alfalfa was correlated with the degree of heterozygosity.

Traditionally, di-haploid lines are generated through microspore cultures. Preparation of microspore cultures is tedious, requires greater expertise and it is genotype specific (Forster and Thomas 2005). In contrast, haploid inducer lines may be used in the breeding program to generate the di-haploid lines. In haploid inducer line system, fertilization results in a subsequent loss of half of the genome set and seed is developed parthenogenetically (Kindiger and Singh 2011). This approach is less genotype-dependent, less laborious and time consuming than the traditional microspore culture technique. USDA-ARS has released two genetic stocks (IL1, IL2) of Italian ryegrass that are characterized by a rapid loss of genome of either parent and seed development through parthenogenesis (Kindiger and Singh 2011). Kindiger and Singh (2011) pollinated the genetic stock of Italian ryegrass with tall fescue to recover di-haploids of tall fescue and Italian ryegrass. Chromosomes underwent a spontaneous doubling to generate highly homozygous lines. In addition to the di-haploids of both species, chimeral sectors, amphidiploid (7 of Italian ryegrass and 21 of tall fescue) and aneuploid were produced. The generated di-haploid lines were screened against artificial and natural selection pressure to identify superior genotypes.

5.8.8 Marker Assisted Selection for Forage Improvement Programs

Genetic mapping has become an indispensable tool for forage improvement programs worldwide. This technique relays on the set of discreetly inherited markers along the entire length of each chromosome of a particular genome. Markers are defined on chromosomes with respect to their distance to each other on a genetic map (Bouton et al. 2012). Genetic mapping of crop species such as wheat and rice is well advanced. In contrast, genetic mapping of only few forage grasses has been completed (Espinoza and Julier 2013). Genome size of many forage crop species is very large and complex which limits the progress in a rapid genome mapping. Genetic mapping of model species such as *Arabidopisis thaliana*, *Medicago truncatula* and *Oryza sativa* could contribute to forage improvement due to homology of gene and genetic order across related species (Espinzoa et al. 2013; Ghamkhar et al. 2012). Syntenic relationships have been observed among subterranean clover, red clover and *M. truncatula* genomes. Candidate genes for isoflavone production in clovers have been identified using *M. truncatula* as a reference genome (Jung et al. 2000). DNA-based markers are widely used in various crop species (Julier et al. 2012). Other marker classes, i.e. morphological and protein markers, are also routinely used for determination of polymorphism in forage species. DNA markers are designed from anonymous DNA or DNA of the analyzed species.

Genetic markers are classified as low or high throughput markers. High throughput marker technologies such as simple sequence repeat (SSR), single nucleotide polymorphism (SNP), sequence characterized amplified region (SCAR) are required

for genetic mapping of a species. In forage species, expressed sequence tags (EST) of specific genes are obtained from various genome data bases and provide information for designing primers for these techniques. ESTs of specific genes in related species are often being used for identification of genes regulating disease and insect resistance in cultivated species (Rubiales et al. 2015). Molecular markers with high throughput capacity have been developed for some forage grasses, i.e. perennial ryegrass, (Wang et al. 2011). Gene targeted SSR markers with di, tri and penta nucleotide motifs of the EST data bases have been used to develop SSR primers (Han et al. 2011; Ramu et al. 2013; Song et al. 2011; Tan et al. 2012). Probe pairs derived from cDNA have been evaluated for development of restriction fragment length polymorphism (Isobe et al. 2003; Sim et al. 2005).

Traits such as disease resistance are monogenic in nature and hence defined as qualitative. Qualitative traits can be identified by a single marker closely linked with gene inducing resistance in plant species. Economical traits linked with forage yield and quality traits are polygenic and defined as quantitative traits. These traits are collectively regulated by several loci within a genome that may interact with each other and affect the mode of gene action, i.e. additive, dominance and epistatic (Kumar 2011). Phenotypic and marker data are integrated to determine the loci of a quantitative trait. Markers linked with particular QTLs are used to select desirable plants in segregating populations. QTLs or major gene selection through the tightly linked DNA-based markers can greatly reduce the time needed to develop a cultivar by enabling selection for desired traits at the seedling stage. Several quantitative traits including forage yield have been improved through the use of QTL mapping (Tanaka et al. 2013). This technique can reduce the number of selection cycles by reducing the impact of environmental variables and linkage drag phenomenon in various breeding schemes such as bulk, recurrent selection and backcross selection. MAS can help perennial forage grass breeding programs by determining desirable traits related with yield productivity and quality at the seedling stage rather than in adult plants.

5.9 Conclusions and Prospects

Forages are integral components of grassland and pasture agro-ecosystem. They are the major source of feed and nutrition for livestock. As primary producers, they are converted by livestock into secondary production in the food chain. Forage breeding is a complex process involving plant morpho-physiological aspects (perenniality, mode of reproduction, mating systems) and aberrant plant-environment correlations affecting plant performance under various sward conditions. The ultimate aim of forage breeding is to develop cultivars with high and sustainable herbage yield under various management systems. It also encompasses development of cultivars with beneficial impacts on ecosystem functions, animal growth and health. Despite the importance of forages in our ecosystem, a forage seed production system is lacking in most of the developing countries, the rate of genetic gain is slow and

improved cultivars with good forage quality are not available for many species. This chapter addresses challenges for forage producers and breeders due to rapidly diminishing grassland areas and the impact on the biodiversity of grassland ecosystems and their productivity. Approaches to conserve genetic diversity and utilize forage genetic resources in an efficient way as well. Forage breeding should be focused on the improvement of forage persistence, biotic and abiotic resistance seasonal availability, improved quality and increased efficiency. Breeding procedures such as half-sib development, recurrent selection and manipulation of heterosis will continue to play a major role in the development of high yielding open pollinated or hybrid varieties. Efficiency of selection will be improved through exploitation of molecular markers and genomics. MAS could be exploited to improve the genetic gains in the selection of polygenic traits such as forage yield per se, IVDM digestibility, increasing water soluble carbohydrates concentration and reduction of protein degradation in the rumen.

Apart from the genomics, forage breeding will benefit from advanced phenotyping tools such as image analyses and metablomics. These techniques have been exploited to understand the whole biochemical pathway for the development of particular phenotypes. This could help in better understanding the genotype × environment interaction and isolation of genes related to particular process.

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Chapter 6
Breeding vis-à-vis Genomics of Tropical Tree Crops

Padmanabhan M. Priyadarshan

Abstract Tree breeding is thought to have a pragmatic future. In the backdrop of climate change and exponential increase in population, developing plants, especially trees, resilient to unpredictable environmental conditions is more challenging than ever before. The benefits of tree species, especially fruit trees are multifold for the sustenance of humankind. Tree genotypes are selected and breeding values estimated for superior characteristics following long-term and costly field-based progeny trials, where provenance trials, clone trials and full-sib progeny evaluations are the backbone of such experiments. Of late, efforts are on to manipulate functions at the level of gene and genome of tree species. The shift from traditional Sanger sequencing to massive parallel sequencing of millions of short DNA pieces simultaneously through Next Generation Sequencing (NGS) is a major breakthrough in the last decade. Among the DNA markers, microsatellites (SSRs – Simple Sequence Repeats of 2–6 bases), which tend to be highly polymorphic, locus specific and co-dominant are preferred in Marker Assisted Selection (MAS) and genetic linkage map construction. Reverse genetics, gene silencing through RNA interference (RNAi), Transcript profiling (DNA microarrays), Serial Analysis of Gene Expression (SAGE) and Massively Parallel Signature Sequencing (MPSS) are some of the latest techniques to learn more about functional genomics. The future of tree genomics is bright, but only serious investigations can help to accelerate the *systems breeding* that integrates information on gene function ensuring superior genotypes.

Keywords Biotechnology • Crop genomics • Tropical tree breeding • Tree crops • Molecular markers

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6.1 Introduction

There are more than 50,000 edible plant species in the world, of which only a few hundred contribute significantly to human food supplies (FAO 1995). Just 15 crop plants provide 90 % of the world's food energy intake with rice, maize and wheat comprising two-thirds of human food consumption (Ji et al. 2013). These three crops alone are the staples of over four billion people. Rice alone feeds almost half of humanity (FAO 2013). Roots and tubers are important staples for over one billion people in the developing world; accounting for roughly 20 % of the food eaten by half the population of sub-Saharan Africa (Alexandratos 2006). Cassava is another major staple food in the developing world, providing a basic diet for around 800 million people (Nassar and Ortiz 2010). Roots and tubers are high in carbohydrates, calcium and vitamin C, but low in protein. An unforeseen consequence of the Green Revolution that prevented large-scale starvation was the rapid rise in micronutrient malnutrition due to lack of adequate vitamins, minerals and fibers in these staple crops (Welch and Graham 1999).

Globally, malnutrition, including nutrient deficiencies and diet-related chronic diseases (e.g. heart disease, cancer, stroke and diabetes), is responsible for more deaths than any other cause, accounting for >20 million mortalities annually (Kennedy et al. 2003; WHO and FAO 2003). Malnutrition also contributes to increased morbidity, disability, stunted mental and physical growth, and reduced national socioeconomic development (FAO 2013). Micronutrient malnutrition alone afflicts more than two billion people, mostly among resource-poor families in developing countries, with Fe, I, Zn, and vitamin A deficiencies most prevalent (Kennedy et al. 2003). Nearly ten million childhood deaths occur from micronutrient malnutrition every year (Black et al. 2003). Leading global economists have identified investing in strategies to reduce malnutrition as the most cost-effective investments governments can make (Anonymous 2008). Consumption of fruits can reduce malnutrition to a larger extent.

Eating fruits may reduce the risk of heart disease, including heart attack and stroke (Dauchet et al. 2006). Fruits may render protection against certain types of cancers. Fruits may reduce the risk of heart disease, obesity and type 2 diabetes. Fruits may also reduce the risk of developing kidney stones and help to decrease bone loss. Most fruits are naturally low in fat, sodium and calories and none have cholesterol. Fruits are sources of many essential nutrients that are under consumed, including potassium, dietary fiber, vitamin C, and folate (folic acid). Fiber in fruits is important for proper bowel function. It helps to reduce constipation and diverticulosis. Vitamin C in fruits is important for growth and repair of all body tissues, helps heal cuts and wounds, and keeps teeth and gums healthy. Folate (folic acid) helps the body form red blood cells.

Reviews featuring the interface between breeding and genomics, especially in trees are very scanty (Priyadarshan and Schnell 2012). Breeding and genomics are expected to work hand-in-hand. However, often these two vital subjects take a parallel course that needs to be amalgamated to achieve penultimate goals in the form

of improved genotypes with better yield and secondary attributes. This chapter is an earnest attempt to bring together the progress of conventional breeding and genomics, and their interactions so as to take stock of the current status and future prospects of these subjects that calls for a unified approach.

6.2 Conventional Breeding

Fruits mostly come from trees. There are an estimated 60,000 tree species on earth (Neale et al. 2013). The genetic attributes of trees stand in stark contrast to those of domesticated annual crops. Trees typically have long generation time with breeding behavior encompassing cross/self-pollinations and incompatibility factors that make the breeding system complicated and their recalcitrance poses yet another bottleneck to breed them suiting to human needs. The quality and endurance of the wood of forest trees, the edibility of fruits and nuts of tree crops and the utility of industrial tree crops make them largely insurmountable for breeding. Traditional breeding involves provenance tests, half/full-sib progeny evaluations, evaluation of clones/ramets, derivation of hybrids and their testing (Priyadarshan 2014). Individual trees are highly heterozygous and thus carry a high genetic load such that mating between related individuals results in inbreeding. While the forest tree species need to be improved as a population, the cultivated tree crops require improvement to derive varieties for seed borne species and clones for species that calls for vegetative propagation. Largely, commercially cultivated crops need improvement of breed or inbred lines (Fig. 6.1). Clonally multiplied species, on the other hand, follow a different pattern for their improvement (Fig. 6.2). Furthermore, unlike crop species, trees are expected to have minimal population sub-structure and low linkage disequilibrium (González-Martínez et al. 2006; Ingvarsson 2005). A practical consequence of low linkage disequilibrium is that linkage relationships between markers and alleles of genes controlling phenotypic traits are not consistent among individuals, which limits the application of marker-assisted selection and breeding.

Traditional tree breeding is a lengthy process that cannot efficiently capture non-additive genetic variation, primarily because inbred lines would suffer from inbreeding depression. Clonal propagation of elite genotypes allows for the capture of both additive and non-additive genetic variation, and the addition of transgenes can confer new or enhanced traits. For example, damage from introduced diseases and insects for which there is no natural genetic basis for resistance could be mitigated through introduction of transgenes conferring resistance (Adams et al. 2000). However, research on the strategies and risks of introducing transgenics into natural populations is still in its infancy (DiFazio et al. 2004; Van Frankenhuyzen and Beardmore 2004). Political, societal and regulatory restrictions make the application of transgenics to trees in the near future uncertain (Herrera-Estrella et al. 2005).

The traditional tree breeding process typically relies on identifying trees with desirable attributes, followed by indirectly evaluating their breeding potential by measuring phenotypic traits in their progeny. Most traits of interest to forest industry

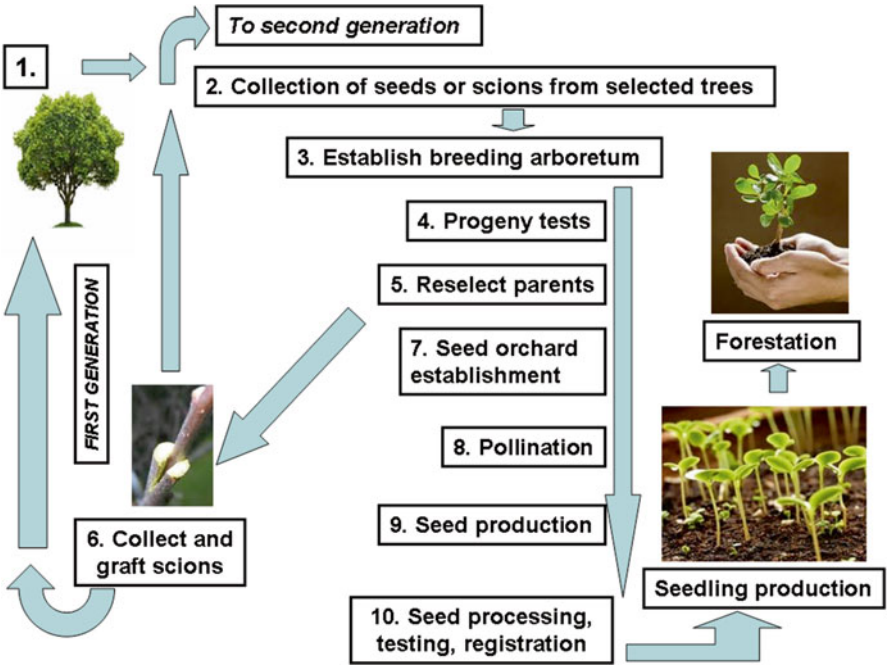

Fig. 6.1 Breeding schemes for seed borne species

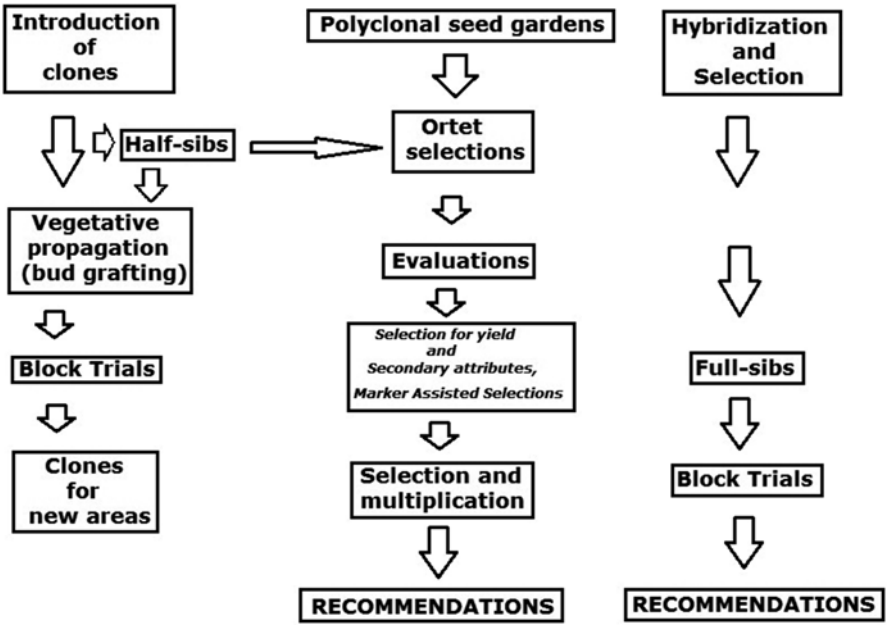

Fig. 6.2 Breeding schemes for vegetatively multiplied species

are quantitative in nature, can be costly to measure, and occur later in development (e.g. wood quality). To better understand the genetic regulation of quantitative traits and speed up the progeny testing process, research has focused on the ability to detect chromosomal regions carrying favorable alleles controlling quantitative traits, so-called quantitative trait loci (QTL). Studies on tree species have demonstrated the feasibility of this approach within pedigrees, and have identified quantitative trait loci influencing traits ranging from wood properties to adaptive traits (Jermstad et al. 2003). Marker-assisted selection is an extension of QTL technology, in which, progeny with desired genotypes within a given pedigree are identified using molecular markers linked to favorable QTL alleles. However, QTL and marker-aided selection have limited application outside of pedigreed material. Limitations to QTL and marker-aided selection are exposed when consideration is given to the low linkage disequilibrium and high allelic variation present, especially in forest tree populations (Brown et al. 2004; Neale and Savolainen 2004). Although linkage relationships between markers and QTLs can be established within pedigrees resulting from controlled crosses, historical recombination between markers and the QTL within populations means that QTL marker relationships must be reestablished in each new pedigree examined, and are completely uncertain in unrelated individuals taken from natural breeding populations.

The molecular analysis of plants is often focused on the single gene level. But the recent technological advances have changed this paradigm. The way the genes and genetic information are organized within the genome and the methods of collecting and analyzing this information, and the determination of their biological functionality is referred to as *genomics*. Genomic approaches are permeating every aspect of plant biology, and since they rely on DNA-coded information, they expand molecular analyses from a single to a multispecies level. Plant genomics is reversing the previous paradigm of identifying genes behind biological functions and instead focuses on finding biological functions behind genes. It also reduces the gap between phenotype and genotype. It is worthwhile to note that these technologies although extensively used in annual agricultural species, are only used in forest tree species research. Except for some tropical (avocado, mango, papaya and citrus) and temperate (apple, prunus and pyrus) fruit species, these techniques are not extensively used in other tree crops.

Tropical fruit trees are a major source of carbohydrates and vitamins. Lack of political stability, resources and infrastructure in tropical regions has lessened the progress of tropical tree genomics compared to temperate species. Developing countries produce 98 % of the tropical fruits. The production of the top three tropical fruit trees, mango, papaya and avocado, is estimated to be 30.7, 12.4 and 3.1 million mt, respectively. Lychee, durian, rambutan and guava are minor tropical fruits. Compounds with a vast number of applications are present in tropical fruit trees. While *Garcinia mangostana* can accumulate up to 56 % oil in seeds (Hawkins and Kridl 1998), fruits of *Mangifera indica*, *Carica papaya* and *Psidium guajava* are the source of carotenoids and vitamin C (Oliveira et al. 2010). Anticancer activity has been described for several tropical fruit tree species like *Irvingia malayana* (Ng et al. 2010) and compounds from *Annona* spp. arrest cancer cells at G1 (Yuan

et al. 2003). Effective gastroenteritis control by *Spondias purpurea* (Caceres et al. 1993) and stimulation of the immune system by *Morinda citrifolia* (Palu et al. 2008), are other fine examples.

Breeding tropical fruit trees is complicated by their reproductive biology. For instance, avocado (*Persea americana*) has an unusual flower behavior with two complementary types of flowering patterns, called A and B, that promotes outcrossing (Bergh 1969). Avocado does not contain any self-incompatibility system and self-pollination is frequently observed. A mature tree can produce upwards of a million flowers with only 1 % of these setting fruit. A single ovary develops into the seed from a single pollination, so the generation of large numbers of seedlings by hand pollination is not practical. In mango (*Mangifera indica*) a similar situation exists, flowering is strongly influenced by weather and some genotypes flower very irregularly. The flowers of mango are small and both perfect and staminate (male) flowers occur on the same inflorescence. Hand pollination is possible but difficult to perform on a large scale to generate a large number of progeny (Pinto et al. 2004). In addition, polyembryony in mango complicates breeding schemes. In polyembryonic cultivars, seedlings arise from nucellar tissue or from a zygote, but distinguishing between the two can be complicated (Schnell and Knight 1992). For many of the tropical fruit tree species in the Sapindaceae, flower polymorphisms (i.e., monoecy, dioecy, adrodioecy, gynodioecy, etc.) add complexity to the breeding process. In lychee (*Litchi chinensis*), there are three types of flowers appearing in irregular sequence or simultaneously on the same inflorescence. There are male flowers, hermaphrodite flowers that act as females and hermaphrodite flowers that act as males (Morton 1987). Again, the flowers are small and not amenable to hand pollination (Stern and Gazit 2003). In longan (*Dimocarpus longan*) there are three flower types, staminate (functionally male), pistillate (functionally female) and hermaphroditic (bisexual). Flowering in each panicle occurs in progressive openings of staminate (male) flowers first, then pistillate flowers followed by hermaphroditic flowers functioning as females and then hermaphroditic flowers functioning as males. Pollination is mainly by small insects, but also by wind (Blanche et al. 2006). Lack of genetic diversity in mangosteen (*Garcinia mangostana*) is a consequence of its mode of reproduction as an obligate apomict. Only anecdotal reports of male trees have been made for this dioecious species (Sand et al. 2005). In addition, an extremely long juvenile stage, upwards of 10 years, has been described for this particular fruit tree species (Poerwanto 2002). Since controlled pollinations are difficult, breeders have relied on open pollinated progeny (half-sib families) for selection. Identifying full-sib families, on the other hand, is relatively simple using SSR markers. Such molecular markers have greatly accelerated mango and avocado breeding efforts.

Hevea rubber (*Hevea brasiliensis*) is one industrial tree species where much progress has been attained in terms of genomics (Priyadarshan 2011). Hevea rubber produces latex in specialized cells known as laticifers or latex vessels, located adjacent to the phloem. These laticifers form a very complex laticiferous system by anastomosis between tubular cells in the tree. Rubber particles are isoprenoid molecules. Isoprenoid biosynthesis is brought about through the mevalonate dependant metabolic pathway (Gronover et al. 2011; Hepper and Audley 1969). Although it is known that biosynthesis

of natural rubber takes place by a mevalonate pathway, molecular biological characterization of related genes has not been adequate. Initial understanding on the regulation of gene expression in the laticifers of *H. brasiliensis* came from the study by Kush et al. (1990), who demonstrated for the first time that transcript levels of genes involved in rubber biosynthesis and genes induced by wounding and ethylene treatment were higher in laticifers than in leaves. Over the years, many studies on genetic transformation (Blanc et al. 2006) and genomics (Saha and Priyadarshan 2012) have added to the information relating to the genetics of Hevea rubber.

6.3 Innovations in Crop Genomics

Plant genomes are best described in terms of genome size, gene content, extent of repetitive sequences and polyploidy/duplication events. Although plants also possess mitochondrial and chloroplast genomes, their nuclear genome is the largest and most complex. There is extensive variation in nuclear genome size (Table 6.1) without obvious functional significance of such variation (Rafalski 2002).

Plant genomes contain various repetitive sequences and retrovirus-like retrotransposons containing long terminal repeats and other retroelements, such as long interspersed nuclear elements and short-interspersed nuclear elements (Kumar and Bennetzen 1999). Retroelement insertions contribute to the large difference in size between collinear genome segments in different plant species and to the 50 % or more difference in total genome size among species with relatively large genomes, such as maize. They contribute a smaller percentage of genome size in plants with smaller genomes such as *Arabidopsis* (Arabidopsis Genome Initiative 2000). If other repetitive sequences are accounted for, the maize genome is comprised of over 70 % repetitive sequences and of 5 % protein encoding regions (Meyers et al. 2001).

Table 6.1 Nuclear genome size in plants

Common name	Nuclear genome size[a]
Wheat	15,966
Onion	15,290
Garden pea	3,947
Maize	2,292
Asparagus	1,308
Tomato	907
Sugar beet	758
Apple	743
Common bean	637
Cantaloupe	454
Grape	483
Man	2,910

[a]Expressed in megabases (1 Mb = 1,000,000)

It is widely accepted that 70–80 % of flowering plants are the product of at least one polyploidization event (Barnes 2002). Many economically-important plant species, such as maize, wheat, potato, and oat are either ancient or more recent polyploids, comprising more than one, and in wheat, three different homologous genomes within a single species. Duplicated segments also account for a significant fraction of the rice genome (Yuan et al 2005). About 60 % of the *Arabidopsis* genome is present in 24 duplicated segments, each more than 100 kilobases (kb) in size (Bevan et al. 2001). Ancestral polyploidy contributes to create genetic variation through gene duplication and gene silencing. Genome duplication and subsequent divergence is an important generator of protein diversity in plants.

6.3.1 Model Plant Species

Model organisms (*Drosophila melanogaster*, *Saccharomyces cerevisiae*) provide genetic and molecular insights into the biology of more complex species. Since the genomes of most plant species are either too large or too complex to be fully analyzed, the plant scientific community has adopted model organisms. They share features such as being diploid and appropriate for genetic analysis, being amenable to genetic transformation, having a (relatively) small genome and a short growth cycle, having commonly available tools and resources, and being the focus of research by a large scientific community. Although the advent of tissue culture techniques fostered the use of tobacco and petunia, the species now used as model organisms for mono- and dicotyledonous plants are rice (*Oryza sativa*) and *Arabidopsis* (*Arabidopsis thaliana*), respectively.

Arabidopsis, a small Cruciferae plant without agricultural use, sets seed in only 6 weeks from planting, has a small genome of 120 Megabases (Mb) and only 5 chromosomes. There are extensive tools available for its genomic analysis, whole genome sequence, Expressed Sequence Tags (ESTs) collections, characterized mutants and large populations mutagenized with insertion elements (transposons or the T-DNA of *Agrobacterium*). *Arabidopsis* can be genetically transformed on a large scale with *Agrobacterium tumefaciens* and biolistics. Other tools available for this model plant are saturated genetic and physical maps.

Unlike *Arabidopsis*, rice is one of the world's most important cereals. More than 500 million mt of rice is produced each year, and it is the staple food for more than half of the world's population. There are two main rice subspecies. *Japonica* is mostly grown in Japan, while *indica* is grown in China and other Asia-Pacific regions. Rice also has very saturated genetic maps, physical maps, whole genome sequences, as well as EST collections pooled from different tissues and developmental stages. It has 12 chromosomes, a genome size of 420 Mb, and like *Arabidopsis*, it can be transformed through biolistics and *Agrobacterium tumefaciens*. Efficient transposon-tagging systems for gene knockouts and gene detection have not yet become available for saturation mutagenesis in rice, although some recent successes have been reported.

6.3.2 Genetic and Physical Maps

The development of molecular markers has allowed for constructing complete genetic maps for most economically important plant species. They detect genetic variation directly at the DNA level. A myriad of molecular marker systems are available, but their description lies beyond the scope of this paper. A genetic map represents the ordering of molecular markers along chromosomes as well as the genetic distances, generally expressed as centimorgans (cM), existing between adjacent molecular markers. Genetic maps in plants have been created from many experimental populations, but the most frequently used are F_2, backcrosses and recombinant inbred lines. Although taking longer to develop, recombinant inbred lines offer a higher genetic resolution and practical advantages. Once a mapping population has been created, it takes only few months to produce a genetic map with a 10 cM resolution. Genetic maps contribute to the understanding of how plant genomes are organized and once available, they facilitate the development of practical applications in plant breeding, such as the identification of Quantitative Trait Loci and Marker Assisted Selection. Most economically important plant traits such as yield, plant height and quality components exhibit a continuous distribution rather than discrete classes and are regarded as quantitative traits. These traits are controlled by several loci each of small effect and different combinations of alleles at these loci can give different phenotypes.

Quantitative Trait Loci analysis refers to the identification of genomic regions associated with the phenotypic expression of a given trait. Once the location of such genomic regions is known they can be assembled into designer genotypes, i.e., individuals carrying chromosomic fragments associated with the expression of a given phenotype. The most important feature of Marker Assisted Selection is that once a molecular marker genetically linked to the expression of a phenotypically interesting allele has been detected, an indirect selection for such an allele based upon the detection of the molecular marker can be accomplished, since little or any genetic recombination will occur between them. Therefore, the presence of the molecular marker will always be associated with the presence of the allele of interest.

Genetic maps are also an important resource for plant gene isolation, as once the genetic position of any mutation is established, it is possible to attempt its isolation through positional cloning (Campos-de Quiroz et al. 2000). Furthermore, genetic maps help establish the extent of genome colinearity and duplication between different species.

Although genetic maps provide much-needed landmarks along chromosomes, they are still too far apart to provide an entry point into genes, since even in model plants the kilobases per centimorgan (kb/cM) ratio is large, from 120 to 250 kb/cM in *Arabidopsis* and between 500 and 1,500 kb/cM in maize. Therefore, a 1 cM interval may harbor ~30–100 or even more genes. Physical maps bridge such gaps, representing the entire DNA fragment spanning the genetic location of adjacent molecular markers.

Physical maps can be defined as a set of large insert clones with minimum overlap encompassing a given chromosome. Initially, generation physical maps in plants were based on YACs (Yeast Artificial Chromosomes). Chimaerism and stability issues, however, dictated the development of low copy, *Escherichia coli*-maintained vectors such as Bacterial Artificial Chromosomes (BACs) and P1-derived artificial chromosomes. Although BAC vectors are relatively small (molecular weight of BAC vector pBeloBAC11 is 7.4 kb for instance), they carry inserts between 80 and 200 kb on average and possess traditional plasmid selection features such as an antibiotic resistance gene and a polycloning site within a reporter gene allowing insertional inactivation. BAC clones are easier to manipulate than yeast-based clones. Once a BAC library is prepared, clones are assembled into contigs using fluorescent DNA fingerprint technologies and matching probabilities. Physical and genetic maps can be aligned, bringing along continuity from phenotype to genotype. Furthermore, they provide the platform clone-by-clone sequencing approaches rely upon. Physical maps provide the bridge needed between the resolution achieved by genetic maps and that needed to isolate genes through positional cloning.

6.3.3 Genome Colinearity/Genome Evolution

A remarkable feature of plant genomics is its ability to bring together more than one species for analysis. The comparative genome mapping of related plant species has shown that the organization of genes is highly conserved during the evolution of members of taxonomic families. This has led to the identification of genome colinearity between the well-sequenced model crops and their related species (e.g. *Arabidopsis* for dicots and rice for monocots). Colinearity overrides the differences in chromosome number and genome size and can be defined as conservation of gene order within a chromosomal segment between different species. A related concept is synteny, which refers to the presence of two or more loci on the same chromosome whether they are genetically linked or not. Colinear relationships have been observed among cereal species (maize, wheat, rice, and barley), legumes (beans, peas and soybeans), pines and Cruciferae species (canola, broccoli, cabbage, *Arabidopsis thaliana*). Recently, the first studies at the gene level have demonstrated that microcolinearity of genes is less conserved; small-scale rearrangements and deletions complicate microcolinearity between closely-related species. For instance, although a 78-kb genomic sequence of sorghum around the locus *adh1* and its homologous genomic fragment from maize showed considerable microcolinearity and the fact that they share nine genes in perfect order and transcriptional direction, five additional, unshared genes reside in this genomic region (Tikhonov et al. 1999).

Comparing sequences of soybean and *Arabidopsis* demonstrated partial homology between two soybean chromosomes and a 25 cM section of chromosome 2 from *Arabidopsis* (Lee et al. 2001). Although such relationships need to be assessed on a case-by-case basis, they reflect the value *Arabidopsis* and other model species offer to economically important species.

Colinearity has also been established between rice and most cereal species, allowing the use of rice for genetic analysis and gene discovery in genetically more complex species, such as wheat and barley (Shimamoto and Kyozuka 2002). A comparison of rice and barley DNA sequences from syntenic regions between barley chromosome 5H and rice chromosome 3 revealed the presence of four conserved regions, containing four predicted genes. General gene structure was largely conserved between rice and barley (Dubcovsky et al. 2001). A similar comparison between maize and rice, based on 340 kb around loci *adh1* and *adh2*, showed five colinear genes between the two species, as well as a possible translocation on *adh1*. Rice genes similar to known disease resistant genes showed no cross-hybridization with maize genomic DNA, suggesting sequence divergence or their absence in maize (Tarchini et al. 2000). There are even reports of colinearity across the monodicotyledoneous division involving *Arabidopsis* and cereals which diverged as far back as 200 million years ago (Mayer et al. 2001). Exploiting colinearity helps to establish cross-species genetic links and also aids in the extrapolation of information from species with simpler genomes (i.e. rice) to genetically complex species (maize, wheat). Furthermore, it reflects the power of genomics to integrate genetic information across species.

6.3.4 Whole Genome Sequencing

Genetic and physical maps at the inter- or intra-species level represent a key layer of genomic information. However, sequence data represents the ultimate level of genetic information. Three major breakthroughs have allowed the sequencing of complete genomes: (1) The development of fluorescence-based DNA sequencing methods that provide at least 500 bases per read; (2) The automation of several processes such as picking and arraying bacterial subclones, purification of DNA from individual subclones and sample loading among others; and (3) The development of software and hardware able to handle massive amounts (gigabytes) of data points.

There are two main approaches to large-scale sequencing (Fig. 6.3). In clone-by-clone strategies, large insert libraries, such as those based on BAC clones, are used as sequencing templates, and inserts are arranged into contigs using diverse fingerprinting methods to establish minimal tiling paths. Sequence Tagged Connectors extracted from large insert clones as well as FISH (Fluorescent in situ Hybridization) and optical mapping are used to extend contigs and close gaps (Marra et al. 1997). BAC clones from sequence-ready contigs are then fragmented into plasmid or M13 vector-based shotgun libraries with insert sizes of ~1–3 kb. Using more than one vector system reduces cloning bias issues. Sequencing efforts are tailored to the degree of coverage required. For instance, for a fivefold coverage, and assuming 500 base pairs (bp) per sequencer reading, 800 clones are sequenced to cover an 80 kb BAC clone. Finished sequences are those obtained at a ~8–10 fold coverage and provide >99.99 % accuracy, whereas working draft sequences are attained at a

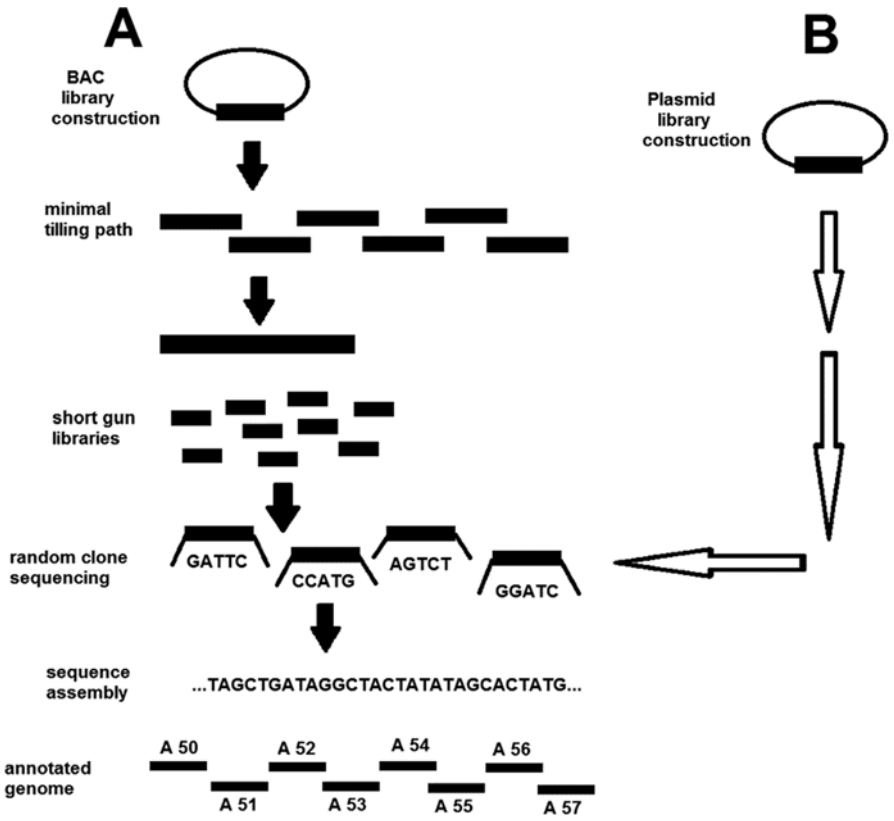

Fig. 6.3 Main approaches to large-scale sequencing

~3–5 fold coverage. It is important to note, however, that even working draft sequences provide an enormous amount of information, and even shotgun approaches rely to some extent on clone-by clone information.

After sequencing is concluded, DNA data is used to reassemble BAC clones. Base calling programs assigning quality scores to each read base such as Phred (Ewing et al. 1998), sequence assembly programs such as Phrap (Gordon et al. 1998), and graphical viewing tools are used to achieve such assembly. The finishing of the sequence then ensues, which can be done in part manually or with finishing software such as Autofinish (Gordon et al. 2001; Gordon and Desmarais 2001).

Annotation, or the process of identifying start and stop codons and the position of introns that permits the prediction of biological function from DNA sequence, proceeds through three main steps. The first is to use gene finders like Xgrail (Uberbacher and Mural 1991) or others based on generalized hidden Markov models, such as GeneMark.hmm (Lukashin and Borodovsky 1998) and GenScan (Burge and Karlin 1997), specifically developed to recognize *Arabidopsis* genes. In the second step, sequences are aligned to protein and EST databases; and finally, puta-

tive functions are assigned to each gene sequence. Successful annotation processes often combine different software and manual inspection.

In shotgun approaches (Fig. 6.3b), which have been successfully used to sequence many microorganisms and *Drosophila melanogaster*, small insert libraries are prepared, and randomly selected inserts are sequenced until a ~5-fold or higher coverage is reached. Sequences are then assembled, gaps identified and closed, and finally annotation conducted. Shotgun sequencing does not rely upon the availability of minimal tiling paths and therefore reduces the cost and effort required to obtain whole genome sequences. Nevertheless, they require an enormous amount of computational power to assembly a large number of random sequences into a small number of contigs. Furthermore, the ultimate quality of large genomes that have been shotgun-sequenced may not be as high as that achievable using the clone-by-clone approach. Because of a high content of long and highly conserved repetitive sequences, including retrotransposons, shotgun sequencing of plant genomes may pose special challenges.

6.3.5 Reverse Genetics

Traditional genetic analysis aims to identify the DNA sequences associated with a given phenotype. Reverse genetics determines the function of a gene for which the sequence is known, by generating and analyzing the phenotype of the corresponding knockout mutant (Maes et al. 1999). Unlike yeast, in which gene disruption is available through homologous recombination, transposon and T-DNA tagging are the best methods available for developing mutagenized plant populations suitable for reverse genetics studies (Pereira 2000). There are several mutagenized populations in *Arabidopsis* suited for reverse genetics studies. A European consortium is developing heterologous systems for rice, based on the Ac element from maize (Greco et al. 2001). There are also proprietary populations such as Pioneer Hi-Bred International's Trait Utility System for Corn (TUSC), mutagenized with the high copy Mu element (Multani et al. 1998). Using high copy elements makes it possible to use smaller populations to ensure that tagged mutants will be found for most genes.

There are two main possibilities for identifying tagged genes at insertion sites. For unknown genes, sequences flanking the insertion can be obtained through inverse Polymerase Chain Reaction (PCR) (Ochman et al. 1988) or Thermal Assimetric Interlaced PCR (Liu and Whitier 1995), whereas for insertions in genes of known sequence, it is possible to amplify and clone the sequence of interest through PCR using gene-specific and insertion-specific primers. Since in the latter case it is common to analyze thousands of plants, PCR-based screening is arranged into three-dimensional pools that allow the unequivocal identification of tagged individuals. Large databases of characterized insertion sites are becoming available that will further ease the use of insertion elements to isolate useful genes (Tissier et al. 1999).

Although several genes have been isolated through reverse genetic approaches, two main factors have limited their wider application. First, many genes are functionally redundant, as even species with simple genomes such as *Arabidopsis* carry extensive duplications, and second, mutations in many genes may be highly pleiotropic, which can mask the role of a gene in a specific pathway (Springer 2000). Nevertheless, reverse genetics is considered to be a major component of the functional genomics toolbox, and it plays an important role in assigning biological functions to genes discovered through large-scale sequencing programs. Transposon tagging provides an excellent alternative to isolate tagged genes that exhibit relatively simple inheritance.

Gene traps refer to another application of transposons that responds to regulatory sequences at the site of insertion. Depending on the sequences engineered, they can be classified as reporter traps, enhancer traps or gene traps. Since they rely on reporter gene expression, mutant phenotypes are not required, and they have been valuable in isolating tissue and cell specific sequences (Springer 2000).

6.3.6 Transcriptional Profiling

While molecular biology generally analyzes one or a few genes simultaneously, recent developments allow the parallel analysis of thousands of genes. This area of genomics involves the study of gene expression patterns across a wide array of cellular responses, phenotypes and conditions. The expression profile of a developmental stage or induced condition can identify genes and coordinately-regulated pathways and their functions. This produces a more thorough understanding of the underlying biology (Quackenbush 2001).

There are several systems available to analyze the parallel expression of many genes such as macroarrays (Desprez et al. 1998), microarrays (Schena et al. 1995) and Serial Analysis of Gene Expression (SAGE) (Velculescu et al. 1995), which consist of identifying short sequence tags from individual transcripts, their concatenation, sequencing and subsequent digital quantitation. SAGE provides expression levels for many transcripts across different stages of development.

There are open and closed transcriptional profiling systems. Open technologies survey a large number of transcripts and analyze their levels between different samples but the identity of the genes involved is not known a priori. One example of such a system is the GeneCalling technology (Bruce et al. 2000). Another open system is provided by Massively Parallel Sequence Signatures (MPSS), where microbeads are used to construct libraries of DNA templates and create hundreds of thousands of gene signatures (Brenner et al. 2000).

Closed systems, on the other hand, analyze genes that have been previously characterized. They include most of the diverse microarray systems available, and these are based on the specific hybridization of labeled samples to spatially separate immobilized nucleic acids, thus enabling the parallel quantification of many specific mRNAs. It is important to select the system at the onset of any transcriptional profiling study and stay with it.

Table 6.2 Basic features of microarrays

Type of DNA	Features
Oligo DNA microarrays (3D-Gene)	An oligo DNA microarray is a DNA microarray whose probe is the chemically synthesized oligo DNA after genes corresponding to exons in the region of 20–100 nts are selected. Since the probe is shorter than a cDNA microarray, its specificity is high and cross hybridization can be inhibited
cDNA microarrays	A cDNA microarray consists of a collection of cDNA fragments which are reverse transcripts of gene transcription products (mRNA). These cDNA fragments are immobilized on a substrate. The probes are the cDNA fragments previously adjusted according to the PCR method. A cDNA microarray is produced by spotting the fragments on the substrate using a spotter. The lengths of cDNA are generally not uniform. Nucleic acid probes for detection can be spotted ranging from a few hundred nucleotides to over a thousand
BAC clone chips	A BAC clone chip is a DNA microarray whose probe is a template amplified by PCR. The template is a genome region incorporated into a comprehensive BAC (bacterial artificial chromosome) clone which various research institutions used in decoding the genome sequence of various organisms. BAC is a vector that can clone a gene of 100–300 kb long. A BAC clone chip is used to analyze the number of genomic DNA copies in such methods as the CGH array

The focus here is on microarrays. In microarray experiments, DNA samples corresponding to thousands of genes of interest are immobilized on a solid surface such as glass slides in a regular array. The immobilized sequences are usually referred to as probes. RNA samples (or their cDNA derivatives) from a biological samples under study are hybridized to the array and are referred to as the target. Labeling with fluorescent dyes with different excitation and emission characteristics allows the simultaneous hybridization of two contrasting targets on a single array (Aharoni and Vorst 2001). Microarrays can be based on oligonucleotides or cDNA molecules, and their basic features are presented in Table 6.2.

Microarray applications are broadly classified as expression-specific and genome-wide expression studies. In specific expression studies, they are used as a functional genomics tool to address the biological significance of genes discovered through large-scale sequencing, as well as a means to understanding the genetic networks explaining biological processes or biochemical pathways. The value of using microarrays to identify novel response genes has been demonstrated by studying the gene expression patterns during maize embryo development (Lee et al. 2002), the response to drought and cold stresses (Seki et al. 2001), herbivory (Arimura et al. 2000) and nitrate treatments (Wang et al. 2000).

When addressing a specific pathway or biological process, it is useful to include genes beyond those of apparent interest, since over-specific microarrays would not be able to address genetic interactions with other biological processes. This principle revealed previously unexpected relationships between low soil phosphate levels and cold acclimation in *Arabidopsis* (Hurry et al. 2000). Genes obtained from the transcriptional analysis of plant responses to stress are of particular relevance for transgenic approaches, as thoroughly reviewed by Dunwell et al. (2001).

Genome-wide arrays are mostly designed for model organisms such as *Arabidopsis* or rice, as there are many genes available to select from, either as clones or as annotated genomic sequences for model species. They are also available to species such as maize that have extensive EST collections. This enabling technology is an immediate and direct result of large-scale sequencing projects. Genome-wide expression profiles are the ultimate tool to integrate all genes existing in an organism into a series of experiments. They also help to elucidate the coordinate expression of different genetic networks and document how changes in one would impact others. It is expected that such genome-wide approaches will be particularly useful in identifying new regulatory sequences and master switches that affect distinct but apparently unrelated genetic networks.

Transcriptional profiling technologies play a central role in predicting gene function since sequence comparison alone is insufficient to infer function. They also help to detect phenomena such as gene displacement – non-homologous genes coding for proteins that serve the same function- and gene recruitment – genes with identical sequences coding for completely different functions (Noordewier and Warren 2001).

Unlike animals, plants cannot move and have developed exquisite mechanisms to cope with changing environmental conditions and biotic challenges, since these directly or indirectly affect most biological processes occurring in plants. Therefore, a significant proportion of the information gathered by specific and genome wide transcription profiling processes should have practical applications and facilitate the development of plants more resilient to biotic and abiotic stimuli. Pérez-de-Castro et al. (2012) have put forth a model genomic selection scheme based on Genomic Estimated Breeding Values (GEBVs) (see Fig. 6.4).

6.4 Genetic Markers and Population Genetics

Molecular genetic markers have been extremely useful for tree-population genetics, a discipline supporting both basic research into the evolution of species and populations, and applications ranging from tree improvement to conservation and restoration. Molecular markers have been used to estimate population parameters including population structure, gene flow, hybridization, migration, mating systems and inbreeding. Knowledge of these attributes can be used to guide applications for management and conservation.

For example, existing marker technologies can be used to determine levels of genetic diversity and inbreeding, two factors indicative of adaptive potential, which can help identify populations at risk. Existing markers can determine taxonomic relationships, a crucial component of establishing the legal basis for protection of endangered plant species. Contamination by non-local seed sources can erode the local adaptation of a population, and can potentially be detected using existing marker technology.

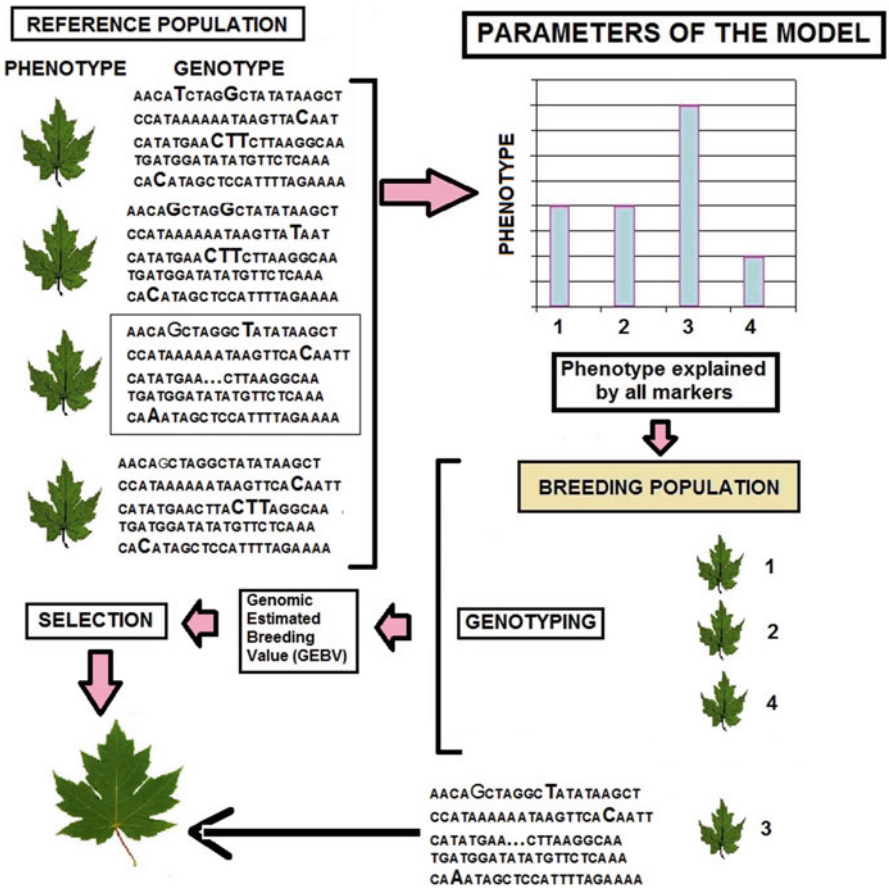

Fig. 6.4 Genomic selection scheme. This model explains phenotype based on all markers analyzed. The model predicts the phenotype of plants in a breeding population on the basis of the genotyping results. This is the genomic estimated breeding value (GEBV), used to select the desired phenotypes

A major limitation of currently-available markers is that they are neutral, meaning they are not within the actual genes that play a causative role in determining traits of interest. In addition, recombination and low-linkage disequilibrium in tree populations means that linkage relationships between markers and alleles of genes controlling phenotypic traits are not consistent among individuals. This is a limiting factor for the application of marker technology to conservation and restoration applications because the markers have little or no predictive value for evaluating adaptive genetic attributes.

Efforts to develop genetic marker-based approaches to breeding forest trees began in the late 1980s. Approaches based on first generation of markers, allozymes, were not feasible due to the very limited number of markers (<50). The first

DNA-based markers, RFLPs, brought more hope as moderately dense genetic maps could be constructed to scan the genome and map quantitative trait loci (QTLs). This approach was quite effective toward mapping QTLs in many forest tree species but the approach could not be brought to application in tree breeding due to low levels of linkage disequilibrium (LD) in forest tree breeding populations and recombination between flanking markers and QTLs with each generation. The next generation of DNA markers based of the polymerase chain reaction (PCR), RAPD, AFLP and SSR, did not solve the LD and recombination problem, even though more markers were available and throughput increased. The situation began to change in the early 2000s with the availability of automated DNA sequencing technology and Single Nucleotide Polymorphism (SNP) genetic markers. Now association studies could be performed where SNPs within candidate genes controlling complex traits could be identified and thus *solving* or minimizing the LD and recombination limitation.

6.4.1 Simple Sequence Repeats (SSRs)

Simple sequence repeats (SSRs) are stretches of 1 to 6 nucleotide units repeated in tandem and randomly spread in the genome. SSRs are very polymorphic due to the high mutation rate affecting the number of repeat units. Such length-polymorphisms can be easily detected on high resolution gels (e.g. sequencing gels), by running PCR amplified fragments obtained using a unique pair of primers flanking the repeat (Weber and May 1989). SSRs have several advantages over other molecular markers. They are: (a) microsatellites allow the identification of many alleles at a single locus, (b) evenly distributed all over the genome, (c) co-dominant, (d) little DNA is required and (e) the analysis can be semi-automated and performed without using radioactivity.

Significant sequence resources in the form of expressed sequence tags are available for numerous trees, with the largest resource being >78,000 transcript assemblies for *Pinus taeda* (loblolly pine). Notably, these resources are being expanded through resequencing of alleles in support of association genetic studies. Examples of additional resources for forest genomics include microarray resources (Abbott et al. 2008), proteomics (Lara et al. 2009), gene tagging and mutant collections (Layne and Bassi 2008). For several angiosperms, transformation systems have been established that enable assessment of gene function using various strategies, including knock down using RNAi (Enrique et al. 2011) or synthetic miRNAs (Song et al. 2010), or introduction of mutations into the amino acid sequence (Pillitteri et al. 2004).

Currently, association genetic studies in trees require a survey sequencing of alleles of candidate genes within a population to identify single nucleotide polymorphisms (SNPs) that define unique gene alleles. SNP genotypes and phenotypes are then measured for individuals sampled from the population, enabling testing for statistical association between SNP genotypes and phenotypes. Furthermore, link-

Table 6.3 ESTs and SSRs of some of the species

Species	Nucleotide	EST	SSR
Carica papaya	51,217	77,393	45+
Citrus spp.	2,592	549,188	106
Persea americana	493	16,558	0
Mangifera indica	401	68	462
Cocos nucifera	382	6	0
Citrus grandis (C. maxima)	202	0	17
Spondias purpurea	141	0	0
Dimocarpus longan	137	66	0
Litchi chinensis	88	0	27
Psidium sp. (guava)	75	0	24
Durio spp.	66	0	7
Garcinia mangostana	64	149	0
Morinda citrifolia	62	0	0
Anacardium occidentale	30	0	21
Tamarindus indica	25	0	0

age disequilibrium decays rapidly within a few hundred base pairs in both pine (Gonzalez-Martinez et al. 2006) and aspen (Ingvarsson 2005). As a result, a SNP with significant association with a phenotypic trait is likely to be close to or in the gene influencing the phenotype. This allows knowledge of gene function to be considered in understanding the genetic mechanisms regulating the trait being evaluated.

SSRs have been the most widely employed class of molecular markers used in genetic studies with applications in many fields of genetics including genetic resources conservation, population genetics, molecular breeding and paternity testing (Ellegren 2004). This range of applications is due to the fact that SSR markers are co-dominant, multi-allelic, highly reproducible, have high-resolution, are amenable to high throughput and are based on polymerase chain reaction (PCR) (Oliveira et al. 2006). As a convention, SSRs are regions in the genome where a group of bases (1–8 bp long) are repeated in tandem (Richard et al. 2008). These regions can be isolated either by data mining of existing sequences or by generating SSR-enriched libraries (Kijas et al. 1994; Zane et al. 2002). With the exception of *Carica papaya* and *Theobroma cacao* for which the genomes have been sequenced (Argout et al. 2011; Ming et al. 2008), most tropical fruit tree species do not have enough DNA sequence information to use data mining for identifying potential markers. Alternatively, expressed sequence tag (EST) information (i.e. cDNA) may be used to develop markers. The number of entries for nucleotides, ESTs and SSRs listed in the National Center for Biotechnology Information (NCBI) database, GenBank, for some of the most important tropical fruit trees are available in Table 6.3.

The use of molecular markers in tropical fruit trees can sometimes be hindered for socioeconomic reasons. One limitation is the significant cost associated with the

development of markers for each crop. Tropical fruit tree species are distributed in a large number of taxonomic groups (Muchugi et al. 2008), and although transferring markers from other species could be used to reduce costs (Viruel and Homaza 2004), such transferability is not feasible among distant taxa (Ellis and Burke 2007). In general, funds to study each tropical fruit tree species are scarce, resulting in insufficient information on molecular markers. Additionally, the practical implementation of the existing results on molecular markers is often limited by the absence of guidance on how to best apply them (Muchugi et al. 2008). For example, from the top 25 indigenous tropical fruit tree species identified as priority by the International Centre for Research in Agroforestry (ICRAF), only for 8 has some work been done using molecular markers (Jamnadass et al. 2009). For most of these species the amount of genetic information available is negligible.

The isolation of SSRs from species for which little to no genetic information is available, such as most tropical fruit trees, can be difficult. At the USDA-ARS Mid South Area Genomics Laboratory (MSAGL) an effective pipeline has been created to isolate SSR markers from these species. The process first involved a slight modification to the DNA extraction method, given the presence of copious latex and phenolics in the vegetative tissues. Second, for generating SSR-enriched libraries, the method previously developed by Techen et al. (2010) was modified to adapt to high throughput pyrosequencing with a Roche 454 GS-FLX (F. Hoffmann-La Roche Ltd, Basel, Switzerland). One modification employs two adapters (Techen et al. 2010) that allow simultaneous loading of pairs of samples in the same region of picotiter plates; the adapters act as bar-coding to separate the samples via bioinformatics. Another modification is reducing the number of PCR cycles during library preparation to minimize redundant sequences. SSRs were isolated from the following species/crop groups: *Nephelium lappaceum* (rambutan), *Manilkara zapota* (sapodilla), *Pouteria sapota* (sapota), *Litchi chinensis* (lychee), *Melicoccus bijugatus* (Spanish lime), *Annona squamosa* (sugar apple), *Dimocarpus longan* (longan), *Averrhoa carambola* (star fruit), *Artocarpus altilis* (breadfruit) and *Garcinia mangostana* (mangosteen).

6.4.2 Single Nucleotide Polymorphism (SNP)

SNP is single-letter change in DNA. SNPs are not considered abnormal; they are simply part of the natural genetic variation within a population that creates diversity. SNPs are part of the reason because it is said that *no two people are alike*, but they occur in a variety of non-human organisms, too. Not all single-letter changes in DNA are SNPs. To be classified as a SNP, at least 1 % of the general population must have that change. Some single-letter changes in DNA have an effect (i.e. substitution of one amino acid for another in the encoded protein), and some have no effect. Researchers find it is valuable to study SNPs because they may be landmarks for pinpointing a disease-causing gene.

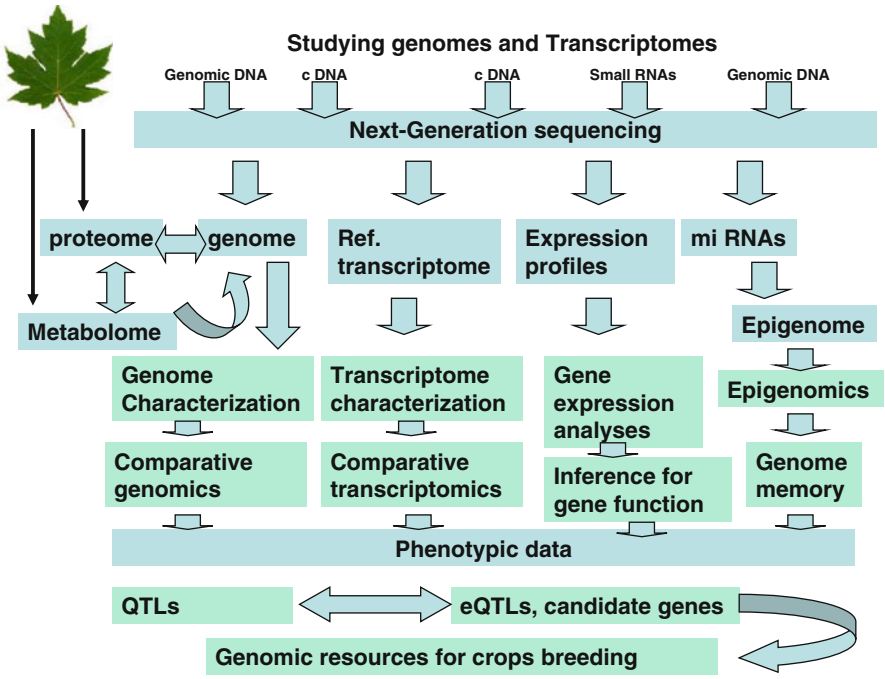

Fig. 6.5 Schemes for studying genomes and transcriptomes

SNP discovery from transcriptome and genomic sequence data is needed to obtain sufficient molecular markers for complete linkage map saturation. The following strategy has been used at SHRS to generate and validate new SNP and SSR markers. The strategy takes advantage of two next generation sequencing platforms, Illumina GAII (Illumina, Inc., San Diego, CA) and Roche 454 and uses both transcriptome and genomic sequence data to identify and validate SSRs and SNPs. Once sufficient SNPs have been identified (5–10 K), the production of an Illumina Infinium oligonucleotide array for genotyping of mapping populations may be employed. Hass, the most important commercially grown avocado cultivar in the world, has been selected as the reference cultivar for the transcriptome sequencing (see Fig. 6.5 for details of studying genomes and transcriptomes).

Morphological markers take years to be usable until the trees overcome their juvenility stage. Molecular markers instead, can be used from any tissue at any time during the plant growth (Azofeifa-Delgado 2006). In tropical fruit tree species for which Expressed Sequence Tags (ESTs) are available, these can be used to develop markers such as SSRs and Single Nucleotide Polymorphisms (SNPs) in relation to flavor, color, fragrance, vitamins, fruit softening and other traits of interest, e.g., *Actinidia* spp. (Crowhurst et al. 2008). SNPs are becoming more popular than SSRs as genetic markers in linkage analysis because they are more abundant and suitable for automatic allele calling (Novelli et al. 2004; Selmer et al. 2009). SNPs have been developed only for a few tropical fruit tree species,

one in relation to resistance to papaya ring-spot virus (PRSV) (Dillon et al. 2006), for phylogenetic studies in *Citrus* spp. (Novelli et al. 2004) and for genotyping and linkage mapping in *Theobroma cacao* (Livingstone et al. 2010). Though developing and testing SNPs is costlier, the same is actively being carried out in avocado and mango. As said earlier, progress with tropical fruit species is meager due to socio-economic-political situations prevailing in tropical countries which are mostly developing and underdeveloped countries. Because of this, case wise treatment is given for tropical species.

6.5 Genomics of Tree Crops

Genomics of many tree crops has been studied in some details. Some of them are: avocado, mango, lychee, cacao, coconut, oil palm, date palm and *Hevea* rubber. Case studies of these are dealt in some detail below.

6.5.1 Avocado

Avocado (*Persea americana*), a subtropical evergreen tree is native from Mexico to northern South America. Global production in 2000 exceeded 2.4 million mt and the major producers were Mexico, Indonesia, South Africa and the USA (Anonymous 2002). Avocado is subdivided into three races: Mexican (*P. americana* var. *drymifolia*, Guatemalan (*P. americana* var. *guatemalensis*) and West Indian (*P. americana* var. *americana*) races. Sterility barriers do not exist between or among the three races (Lahav and Lavi 2002). Avocado is protogynous, diurnally synchronous dichogamy (Berg 1969), promoting out-crossing; however, significant amounts of self-pollination are known to occur in commercial plantings (Borrone et al. 2008; Davenport et al. 1994; Schnell et al. 2009). The haploid genome size of avocado has been estimated to be 8.83×10^8 bp (Arumuganathan and Earle 1991a, b).

There was a limited set of SSR markers for *Persea americana* (Ashworth et al. 2004; Sharon et al. 1997), some of which do not consistently amplify in all varieties (Ashworth and Clegg 2003; Ashworth et al. 2004). For example, 14 of 39 SSR markers tested were suitable to fingerprint diverse collections of *P. americana* (Schnell et al. 2003). To increase the number of informative SSR markers, publicly available *P. americana* ESTs were screened (Borrone et al. 2007, 2009) bringing the number of markers available to upwards of 300. Data-mining SSRs from expressed sequence tags (ESTs) has proven effective for generating markers for fingerprinting, genetic mapping and comparative mapping among species (Varshney et al. 2005).

The first linkage map of avocado was produced by Sharon et al. (1997) from a progeny of a cross between cvs. Pinkerton and Ettinger using 50 SSR markers, 17 Random Amplified Polymorphic DNA (RAPD) markers, and 23 minisatellite DNA Fingerprint (DFP) markers. Twelve linkage groups with 34 mapped loci covering

352.6 centimorgans (cM) were identified. Seven linkage groups contained two markers, two linkage groups contained three markers, one linkage group contained four markers, and two linkage groups contained five markers each. A larger population and an increased number of genetic markers were needed to produce a linkage map useful for quantitative trait loci (QTL) discovery. The development of over 300 SSR markers (Ashworth et al. 2004; Borrone et al. 2007, 2009; Sharon et al. 1997) enabled the development of a partially-saturated genetic linkage map and the potential identification of QTLs controlling horticultural traits of interest in avocado. In California (Ashworth et al. 2007; Chen et al. 2007) and in Spain (Viruel et al. 2007) mapping populations were developed by producing full-sib families that are similar in size to those used by Sharon et al. (1997).

In Florida, a large population of seedlings from a commercial grove inter-planted with two cultivars of opposite flowering types, Tonnage (Type B) and Simmonds (Type A), in approximately equal numbers were screened to determine the outcrossing rate in avocado under south Florida conditions (Borrone et al. 2008). Eight fully informative SSR markers identified 870 seedlings as progeny of a reciprocal cross between Tonnage and Simmonds. Using these seedlings, the first large mapping population and linkage map for QTL discovery were developed in avocado, focusing upon West Indian-Guatemalan hybrids (Borrone et al. 2009). The final linkage map was constructed from 163 markers generated by 135 primer pairs, 112 designed from EST-SSRs and 23 SSR primers developed by Sharon et al. (1997).

6.5.2 Mango

Mango (*Mangifera indica*) is a significant tree fruit crop grown commercially in tropical and subtropical areas of many countries. It has been under cultivation in India for at least 4,000 years and over 1,000 varieties are known to exist in India (Mukherjee 1953). Isozymes were the first markers to be used for fingerprinting mango cultivars, to determine self vs. cross pollination and to estimate genetic relationships (Degani et al. 1990; Knight and Schnell 1994). RAPD markers were also used to fingerprint cultivars and estimate genetic relationships in mango (Schnell et al. 1995). Lopez-Valenzuela et al. (1997) used RAPD markers to estimate genetic diversity among 15 rootstock cultivars using 13 markers, and identified a specific RAPD band associated only with the polyembryonic types. Eiadthong et al. (1999) utilized anchored simple sequence repeat markers to analyze 22 mango cultivars; they were able to distinguish genotypes. However, the authors were unable to find markers unique to either monoembryonic or polyembryonic types, or for the Thai cultivars selected for green harvest (crispy mango) from the cultivars selected for ripe fruit production. Pandit et al. (2007) also used Inter-Simple Sequence Repeat (ISSR) markers to evaluate 60 elite Indian mango cultivars and 10 non-Indian cultivars. They were not able to distinguish Indian cultivars from north and south India and they concluded that ISSR markers could not be considered a comprehensive marker system for mango. Kashkush et al. (2001) utilized Amplified Fragment

Length Polymorphisms (AFLP) to estimate genetic relationships between 16 cultivars and 7 rootstock cultivars. They also analyzed 29 progeny from a cross of Tommy-Atkins and Keitt and produced a crude linkage map that identified 13 of the 20 linkage groups.

Viruel et al. (2005) developed the first reported set of 16 SSR markers for mango, of which 14 produced the expected 1 or 2 amplification products per genotype. These 14 SSRs were used to evaluate 28 mango genotypes that included 14 Florida cultivars. Discrimination of all 28 genotypes was possible and the average number of alleles per locus was 5.3. Previously known pedigree information for the Haden family of mangos was confirmed and was in agreement with published RAPD and DFP analyses (Adato et al. 1995; Schnell et al. 1995). Schnell et al. (2005) developed a second set of 15 SSR markers and analyzed 59 Florida cultivars and 4 related species. Two of the SSRs were monomorphic among the Florida cultivars; the other 13 had an average number of alleles per locus of 4.2 with Polymorphism Information Content (PIC) values varying from 0.21 to 0.63.

Schnell et al. (2006) used 25 SSR loci to estimate genetic diversity among 203 unique mangos (*Mangifera. indica*), 2 *M. griffithii* and 3 *M. odorata* accessions maintained at the National Germplasm Repository (NGR) and at Fairchild Tropical Garden in Miami, Florida. The 25 SSR loci had an average of 6.96 alleles per locus and an average PIC value of 0.552 for the *M. indica* population. The total propagation error in the collection (i.e. plants that had been incorrectly labeled or grafted) was estimated to be 6.13 %. When compared by origin, the Florida cultivars were more closely related to Indian than to Southeast Asian cultivars. Unbiased gene diversity (H_{nb}) of 0.600 and 0.582 was found for Indian and Southeast Asian cultivars, respectively, and both were higher than H_{nb} among Florida cultivars (0.538). When compared by horticultural type, H_{nb} was higher among the polyembryonic types (0.596) than in the monoembryonic types (0.571).

The first genetic linkage map in mango was reported by Kashkush et al. (2001) utilizing AFLP markers and 29 progeny from a cross of Tommy-Atkins x Keitt in Israel. They were able to map 34 AFLP loci and produced a crude linkage map that identified 13 of the 20 linkage groups covering 160 cM. A second map has been produced using 60 progeny from a cross of Keitt x Tommy-Atkins in China using AFLP markers. A total of 81 markers with the correct segregation ratios were identified and 39 of these were used to identify 15 linkage groups. The average distance between two adjacent markers was 14.74 cM. Improvement of the mango recombination map requires the development of more co-dominant molecular markers. Using Roche 454 sequencing and the SSR discovery pipeline discussed earlier in this chapter, new SSR markers have been developed and verified and are now being validated at SHRS on mapping populations. A suitable number of SSR markers are being identified to develop a moderately saturated recombination map for mango.

To develop a marker assisted selection (MAS) program for mango more extensive linkage maps need to be developed and mapping populations field evaluated. The progeny size of both of the Florida mapping populations is small (168 and 224). In the Australian program, sizable populations have been developed from controlled pollination. These populations are the best candidates for QTL identification in

mango. The populations developed from isolated groves by the USDA-ARS SHRS in Florida together with the Australian populations will be used in the next few years to produce a comprehensive linkage map and to identify QTL for disease resistance and for important horticultural traits. Holton (2010) reported that the Queensland Primary Industries and Fisheries has invested in a gene discovery project for mango with the goal of discovering genes controlling consumer and grower traits, such as fruit quality and tree architecture. Using a multi-disciplinary approach they are sequencing expressed genes via ESTs and Serial Analysis of Gene Expression (SAGE), using next generation sequencing producing low pass genome converge, identifying candidate genes from fruit quality and tree architecture, and identifying aroma volatiles from fruit.

6.5.3 Cacao

Cacao was domesticated approximately 3,000 years ago in Central America (Brown and Schnell 2008; Coe and Coe 1996; Henderson et al. 2007). Cacao diseases reduce the potential crop by an estimated 810,000 mt annually (30 % of world production) and individual farm losses can approach 100 % (Bowers et al. 2001; Keane 1992). Cacao production is essential to the livelihoods of 40–50 million people worldwide. Recent molecular analyses have permitted cacao germplasm classification into ten major clusters or groups: Amelonado, Contamana, Criollo, Curaray, Guiana, Iquitos, Marañón, Nanay, Nacional, and Purús (Motamayor et al. 2008). The Matina 1–6 clone is a traditional cultivar that exhibits the Amelonado phenotype and belongs to the Amelonado genetic group. The flow cytometry (FCM) estimate of the Matina 1–6 genome size is 445 Mbp (Motamayor et al. 2013). The International Cacao Genome Sequencing consortium (ICGS) produced a total of 17.6 million 454 single reads, 8.8 million 454 paired end reads, 398.0 million Illumina paired end reads and about 88,000 Sanger BAC end reads, corresponding to 26 Gb of raw data.

Isozymes were the first molecular markers utilized in cacao (Lanaud 1986). Although the available loci and the numbers of polymorphisms typically generated by each isozyme were low, this simple system enabled assessment of genetic diversity and mating system, assisted genotype identification, and contributed to linkage mapping (Ronning and Schnell 1994; Sounigo et al. 2005). However, the isozyme markers are outdated because of their low polymorphism and the environmental effect on the *phenotype*. Commonly used DNA markers in cocoa include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSRs). RFLP was first applied in cocoa in the early 1990s (Laurent et al. 1994). The polymorphism of RFLP is moderately high in cacao. Its high reproducibility and the co-dominant allelic nature is useful for the construction of genetic linkage maps and tagging genes and quantitative trait loci (QTL) linked to characters of agronomic importance (Risterucci et al. 2000) and assessment of genetic diversity (Motamayor et al. 2002). Because RFLP probes are invariably specific to

a limited number of loci, RFLP is not a very effective tool for cacao genotype identification. Another major drawback is that RFLP is not amenable to automation and data generation is both laborious and expensive. RAPD was the first PCR based DNA fingerprinting method to be applied for the genetic characterization of cacao (Wilde et al. 1992). This system is technically simple to perform but has a low reproducibility between experiments and laboratories.

AFLP is a PCR based fingerprinting protocol that combines the strength of RAPD and RFLP that is highly polymorphic with considerable reproducibility within a laboratory (Vos et al. 1995). Because of its dominant nature, AFLP is not a direct measure of heterozygosity and so it has limited use in genotyping. SSRs, also known as microsatellites, have emerged as the most widely used marker for cacao, enabling great strides to be made in the characterization of cacao germplasm (Lanaud et al. 1999). SSRs are typically co-dominant and multiallelic, allowing precise discrimination (or matching) of individual clones based on the multi-locus fingerprints. As SSR sequence information can be easily shared between laboratories, data generated in different laboratories can be compiled and marker-specific size ladders containing all the common alleles for a given locus can be generated (Cryer et al. 2006a, b; Sampaio et al. 2003). The standardized datasets then can be merged for joint analysis (George et al. 2004; Presson et al. 2006).

The first cacao linkage map was developed for the UPA402 × UF676 progeny, containing 193 marker loci (mainly RFLPs and RAPDs), covering 759 cM in 10 linkage groups, corresponding to the haploid chromosome number (Lanaud et al. 1995). The latest published version of this high-density map contained 465 markers, 268 of which were SSRs and 16 resistance gene analogs (RGA), covering 782.8 cM, and it was based on 135 individuals (Pugh et al. 2004).

Cacao genetic maps have been used to detect QTLs for various agronomically-important traits, including resistance to the three major fungal diseases (black pod rot caused by *Phytophthora* species, frosty pod rot caused by *Moniliophthora roreri*, witches'-broom caused by *Crinipellis perniciosa*), yield components, plant vigor and quality traits (Brown et al. 2005; Clement et al. 2003a, b; Flament et al. 2001; Lanaud et al. 1996; Motilal et al. 2002; Queiroz et al. 2003; Risterucci et al. 2003). Black pod rot, is the most important disease of cocoa worldwide. *Phytophthora palmivora* occurs globally, while *P. megakarya* is seen in West Africa, and *P. capsicii* and *P. citrophthora* occur in the Americas. To identify genomic regions associated with resistance to *Phytophthora* species and isolates, 12 linkage maps have been published for populations in Ivory Coast, Costa Rica, Cameroon, Trinidad, and France (Flament et al. 2001).

Unraveling of the gene expression networks underlying growth and development of cacao shows promise. Several cacao EST sequencing projects have resulted in 6,569 ESTs being deposited to date in dbEST (Jones et al. 2002; Verica et al. 2004). ESTs cannot be used simultaneously to compare differences in expression between multiple tissues or between multiple treatments. But, microarrays allow comparative measurement of gene expression levels in thousands of genes in a single experiment. Here, sequences corresponding to specific genes are spotted onto array and labelled RNA from a tissue understudy is then

hybridized to the array. The transcription profiles of all the arrayed genes in a given tissue can be studied like this.

6.5.4 Papaya

The first genetic map of papaya, constructed 70 years ago, consisted of only three morphological markers: sex form, flower color and stem color (Hofmeyr 1939), and the ensuing 50 years failed to produce a more detailed genetic map due to lack of morphological markers. RFLP markers were unsuccessful because the Southern filters of papaya could be used only twice that made the process costly and the polymorphism rate among parental lines of papaya mapping population is very low due to the inbreeding (Kim et al. 2002). RAPD markers led to the second genetic map using 62 RAPD markers (Sondur et al. 1996). Automation with a Li-Cor sequencer of amplified fragment length polymorphisms (AFLP, Vos et al. 1995) made it possible to construct a high density genetic map of papaya. A total of 1,778 AFLP markers was generated leading to the construction of the third genetic map of papaya consisting of 1,498 AFLP markers, the papaya ringspot virus coat protein marker, morphological sex type and fruit flesh color (Ma et al. 2004). These markers were mapped onto 12 linkage groups covering a total length of 3,294 cM, with an average distance of 2.2 cM between adjacent markers. Simple sequence repeats (SSR) were mined from the whole genome shotgun sequence and the BAC end sequences of papaya. Over 11,000 SSR markers were surveyed across parental lines SunUp and AU9 used for developing an F2 mapping population. Seven hundred thirteen markers were mapped, including 712 SSR markers and 1 morphological marker (Chen et al. 2007). This fourth genetic map consists of nine major linkage groups corresponding to the nine chromosomes plus three minor linkage groups that initially failed to be integrated.

Five papaya flower cDNA libraries were constructed, three from pre-meiosis (<4 mm) flower buds (male, hermaphrodite, and female) and two from mature flower buds (hermaphrodite and female). ESTs from these five libraries were sequenced from the 5′ end to produce 31,652 clean sequences with a minimum length of 200 nucleotides. The average read length of a clean sequence was 486 nucleotides with a minimum quality score of 20. EST sequences were then clustered based on local similarity scores of pair-wise comparison using 88 % similarity over 100 nucleotides. Clusters containing only one sequence were grouped as singletons. The EST clusters were assembled into contigs (contiguous sequence) by multiple-sequence alignment that generates a consensus sequence for each of the clusters, with criteria of 95 % identity over 30 nucleotide overlap. A unigene set of 8,571 EST contigs and singletons was assembled. A normalized and subtractive cDNA library was constructed using pooled RNA samples isolated from roots, leaves, seeds, Cali, three sex types of flowers, and three ripening stages of fruit. Over 50,000 EST sequences were generated from this library, yielding an additional unigenes with a total of 16,432 unigenes for genome annotation (Ming et al. 2008).

Papaya has been recognized as an excellent model system for studying sex chromosome evolution and for tropical fruit tree genomics. It is a member of the order Brassicales sharing a common ancestor with *Arabidopsis* about 72 mya (Wikström et al. 2001), an excellent out group to study genome evolution in the family Brassicaseae.

The agricultural importance and the unique biological feature of the nascent sex chromosome justified the sequencing of the papaya genome. The transgenic variety SunUp female genomic DNA was chosen for genome sequencing because of its impact on the papaya industry and to avoid complications of genome assembly in the heterozygous male specific region of the Y chromosome. In addition, SunUp's non-transgenic progenitor is Sunset, a Solo variety that has undergone more than 25 generations of inbreeding, an ideal homozygous genotype for a genome sequencing project.

The genome of a SunUp female was sequenced using the whole genome shotgun (WGS) approach with Sanger sequencers (Ming et al. 2008). It was assembled into contigs containing 278 Mb and scaffolds spanning 372 Mb including embedded gaps. The estimated residual heterozygosity of SunUp is 0.06 %, confirming the highly inbred nature of this Solo variety. Of 16,362 unigenes derived from ESTs, 15,219 (92.5 %) matched this assembly. Among 706 BAC end and WGS sequence-derived SSR markers on the genetic map, 652 (92.4 %) could be used to anchor 167 Mb of contigs or 235 Mb of scaffolds to papaya linkage groups in the current genetic map. Papaya chromosomes contain heterochromatin knobs, concentrated in the centromeric and pericentromeric regions. The heterochromatic regions account for approximately 17 % of the genome, representing about 30–35 % of the genomic DNA due to their highly condensed nature. A large portion of the heterochromatic DNA was likely not covered by WGS sequence, as evident by the absence of centromere-specific repeats from the shotgun sequences. The 278 Mb of contig sequence was estimated to represent about 75 % of the papaya genome and more than 90 % of the euchromatic regions, which is in line with the 92.5 % of the EST and 92.4 % of genetic markers covered by the assembled genome.

The assembled genome was masked using a de novo papaya repeat database for genome annotation. Gene predictions were combined with spliced alignments of proteins and transcripts to produce a reference gene set of 27,950 gene models (revised from the 28,038 when the genome sequence was published). A total of 20,067 (71.8 %) of the predicted papaya genes with average length of 1,102 bp shared similarity to proteins in the nonredundant (NR) database from the National Center for Biotechnology Information (NCBI), and 9,642 (48.0 %) of them supported by papaya unigenes. Among 7,971 genes with average length of 307 bp that had no hits to the none-redundant protein database in the GenBank, only 647 (8.1 %) were supported by papaya unigenes, implying that the number of predicted papaya-specific genes was inflated. If the 647 genes with unigene support represent 48.0 % of the total, then 1,348 predicted papaya-specific genes may be real, and the number of predicted genes in the assembled papaya genome would be 21,415. Considering that the assembled genome covers 92.5 % of the unigenes and 92.4 % of the mapped genetic markers, the number of predicted genes in the papaya genome could be 7.5 % higher, or 23,151, about 25 % less than *Arabidopsi*s (Arabidopsis Genome

Initiative 2000; Hanada et al. 2007), 38 % less than rice (International Rice Genome Sequencing Project 2005), 49 % less than poplar (Tuskan et al. 2006), and 24 % less than grape (Jaillon et al. 2007). This number is likely the upper limit for papaya genes, because EST-based unigenes and predicted genes from WGS sequence may each be fragmented and counted multiple times, as demonstrated by initially inflated gene numbers estimated from rice WGS draft sequences.

The papaya genome consists of about 52 % repetitive sequences, including 43.4 % of the papaya genome is homologous to identifiable transposable elements (TEs) and an additional 8.5 % repetitive sequences that are currently unannotated, but are likely to be novel TEs. Most of the >600 types of repeats in Repbase[12] are represented in papaya, with the dominant class being retrotransposons (40 % of the genome) and some of the abundant types being *Ty3-gypsy* (27.8 %) and *Ty1-copia* (5.5 %) retrotransposons. An interesting feature of papaya is the relatively low abundance of known DNA transposons (0.20 %) compared to other plant genomes. The papaya genome is dominated by papaya-specific TE families, accounting for 38 % of the genome sequences.

6.5.5 Palms

The palm family, consisting of over about 2,400 species (Dransfield et al. 2008), is known to botanists as the Arecaceae, although the old name Palmae is still in use. Over 90 % of the diversity within the family is contained within the world's tropics, and the utility of many palms in human industry at both the subsistence and world market levels makes the Arecaceae the third most economically important family of plants after grasses and legumes.

Three palm species account for the large majority of the family's economic importance. *Cocos nucifera* (Harries and Paull 2008) and *Elaeis guineensis* are major export crops throughout the world's tropics, as is the date palm (*Phoenix dactylifera*) in subtropical arid zones. Equally significant are the myriad uses to which local palms are put by indigenous human cultures wherever palms are found naturally (Balick and Beck 1990; Johnson 1998; Schultes and Raffauf 1990). These include exploitation for food, oil, fiber, and construction, as well as medicinal and ceremonial use. The degree to which genomic approaches have been applied towards the improvement of palm tree crops has trailed that of cereals, vegetables, and many temperate fruit crops, probably because oil palm and coconut are crops of the developing world with limited resources for research.

6.5.5.1 Coconut

The coconut (*Cocos nucifera*), is not only the universal symbol of the tropics, but is one of the most important economic plants in the low-latitude developing world. *Cocos nucifera* is pantropically distributed, a present day range significantly

influenced both by a seed well-adapted to oceanic dispersal and the species' importance to humans (Gruezo and Harries 1984; Harries 1978, 1995). Copra, the dried endosperm of the fruit and the source of coconut oil, and coir, the fibers of the mesocarp, are both significant export products for Third World economies (Harries and Paull 2008), and there are many subsistence used for almost all parts of the coconut (Balick and Beck 1990). Coconut cultivars are generally classified into the Tall and Dwarf types. The tall type is primarily out-crossing while the dwarf type, with some exceptions, is mainly selfing. The coconut is diploid, with $2n = 16$ chromosomes (Read 1966). Genome size is estimated at 2C = 6.96 Gbp (Beaulieu et al. 2007).

Marker assisted selection programs in coconut breeding have also not yet been documented, despite as many as six mapping populations worldwide, and a number of QTLs (Ashburner 1999; Baudouin et al. 2005). Nonetheless, large bodies of molecular marker studies of genetic variation, some sequence-based phylogenetic studies, and a few candidate gene and QTL discoveries have been accomplished.

Successive dominant molecular marker applications in coconut have included randomly amplified polymorphic DNA (Ashburner et al. 1997; Cardeña et al. 2003; Manimekalai and Nagarajan 2006a, 2010; Upadhyay et al. 2004; Wadt et al. 1999), organeller RFLPs (LeBrun et al. 1998a, b), AFLPs (Perera et al. 1998; Teulat et al. 2000), inter simple sequence repeats (ISSR) (Manimekalai and Nagarajan 2006b), and inverse sequence-tagged repeats (ISTRs) (Duran et al. 1997; Rohde et al. 1995).

RAPDs have not been used extensively in coconut due to their non-specific nature and low repeatability (Lebrun et al. 2005). ISSR, like RAPD, is a multilocus technique, thus there is the possibility of non-homology of similar sized fragments. It can also have similar reproducibility problems. Little mitochondrial and no chloroplast polymorphism was found in coconut RFLPs derived from these organelles (Lebrun et al. 1998a, b, 1999; Perera 2002). AFLP have been used primarily to more fully saturate genetic maps (Lebrun et al. 2001; Reidel et al. 2009). ISTRs are of greatest use at the population level, and have not been informative for diversity studies across numerous cultivars (Lebrun et al. 2005).

Co-dominant molecular markers include RFLPs from the nuclear genome (LeBrun et al. 1998a, b, 1999), microsatellites (simple sequence repeats, SSR), and single nucleotide polymorphisms (SNPs). The only SNPs developed for coconut were those of Mauro-Herrera et al. (2006, 2007), visualized with single strand conformational polymorphisms (SSCP). SNPs shall get focused on with the advent of next generation sequencing systems.

Three groups have developed SSR primers from *Cocos nucifera* (Karp 1999; Perera et al. 1999, 2000; Rivera et al. 1999; Teulat et al. 2000). Microsatellites have been the most widely utilized molecular markers for diversity analysis in coconut (Dasanayaka et al. 2009; Devakumar et al. 2006; Karp 1999; Martinez et al. 2010; Mauro-Herrera et al. 2007, 2010; Meerow et al. 2003; Perera et al. 1999, 2000, 2003; Rivera et al. 1999; Teulat et al. 2000; Zizumbo-Villarreal et al. 2006). Based on the results of these studies, Lebrun et al. proposed two major cultivar groups: the

Pacific group with five sub-groups (Southeast Asia, Melanesia, Micronesia, Polynesia and the Pacific coast of Central and South America), and the Indo-Atlantic group, originating in India and subsequently dispersed to West Africa, the Atlantic coast of Latin America and East Africa (Lebrun et al. 2005).

Diversity studies have strongly supported a common ancestry/domestication region for dwarfs from India, Malaysia and the Philippines (Devakumar et al. 2006; Harries et al. 2004; Lebrun et al. 1998a, 2005; Mauro-Herrera et al. 2010; Meerow et al. 2003; Perera et al. 2003; Teulat et al. 2000; Upadhyay et al. 2004), all of which appear to be heavily self-pollinating and thus inbred (Meerow et al. 2003; Teulat et al. 2000). The South Pacific Niu Leka dwarf is the exceptional case, as it is much more heterozygous and out-bred (Lebrun et al. 2005; Meerow et al. 2003) and is thought to represent a separate domestication event (Harries 1978).

The first coconut linkage map was constructed in the Philippines using hybrids of a Malayan Yellow Dwarf (MYD) x Laguna Tall (LAGT) cross (Rohde et al. 1999). A total of 382 markers were placed on 16 linkage groups, and 6 QTLs for early flowering were identified (Herran et al. 2000). The total map length was 2,226 cM. Ritter et al. (2000), using the same mapping population, mapped QTLs for leaf production and girth height. The second linkage map was based on a MYD x Rennell Island Tall in Ivory Coast, with 227 markers in 16 linkage groups. Nine QTLs associated to with fruit yield were found (Lebrun et al. 2001). A total length of about 2000 cM was assigned to the coconut genome (Herran et al. 2000; Lebrun et al. 2001). This second mapping population, with the addition of 53 new SSR markers was later used to identify another 52 QTLs for 11 fruit traits including fruit component weight, endosperm moisture content and fruit production (Baudouin et al. 2006).

MicroRNAs are a group of small (20–24 nt), endogenously expressed, non-coding RNAs that play important regulatory roles in plants and animals. So far, more than 800 miRNAs have been identified from angiosperms (Hewezi et al. 2008). miRNAs function by targeting mRNA for cleavage or translational repression and thus affect many functions in plants including leaf (Ori et al. 2007), shoot and root (Guo et al. 2005), and floral development (Allen et al. 2005), as well as stress response (Sunkar et al. 2006). Li et al. (2009) compared the expression profiles of miRNAs between two different stages in the development of endosperm in coconut. They found 32 miRNAs that showed differential expression, thus implicating miRNAs in coconut endosperm development.

With the advent of new DNA sequencing platforms that achieve an ever-increasing degree of speed, coverage and sharply decreasing costs for obtaining the data, we can expect to see many coconut transcriptome libraries, SNP chips, and, ultimately, a complete genome sequencing effort. A coconut bacterial artificial chromosome (BAC) library was reported to exist at CIRAD, with a total length of five times that of the coconut genome (Baudouin et al. 2005), but no publications have as yet appeared utilizing this important genomic tool.

6.5.5.2 Date Palm

The date palm (*Phoenix dactylifera* L.) is one of the oldest fruit crops grown in the arid regions of the Arabian Peninsula, North Africa, and the Middle East (Chao and Krueger 2007, 2008). The most probable area of origin of the date palm was in or near what is now the country of Iraq, but date cultivation spread to many countries beginning in ancient times. Dates are a major food resource and income source for local populations in the Middle East and North Africa, and play significant roles in the economy, society and environment in these areas (Chao and Krueger 2007, 2008). In addition to serving directly as a food, dates are packed and processed in a number of ways, and other parts of the tree are used for various purposes.

Studies have been performed in date palm with DNA-based marker systems. Due to the difficulties in extraction of high quality DNA, RFLPs did not prove suitable for large-scale use in field analysis, and RAPDs became more prevalent in DNA-based genetic analysis of date palms (Bouchireb and Clark 1997). Various other reports of cultivar identification using RAPD have been published (Ben Abdallah et al. 2000; Saker and Moursy 1999; Sedra et al. 1998). The first widely disseminated report on their use in date palm genetic analysis was Cao and Chao (2002). Diaz et al. (2003) used five primer sets generating 310 AFLP fragments to analyze date palm varieties maintained at the Estación Phoenix in Elche, Spain. The technique was useful in analyzing varietal identity and the integrity of tissue-culture derived plantlets. Jubrael et al. (2005) used AFLP to analyze date palm germplasm from Iraq. A total of 122 polymorphic AFLP loci were observed, with an average of 17.4 polymorphic loci per primer combination. The use of any of the 4 combinations was sufficient to identify all 18 of the varieties studied. The varieties were separated into two groups based upon their genetic relationships, which ranged from moderate to diverse.

Microsatellite markers became a preferred technique for genetic analysis of date palms. Most of the efforts with microsatellites have involved varietal identification, with some work in the area of genetic analysis and phylogeny. Billotte et al. (2004) constructed a $(GA)_n$ microsatellite-enriched library and characterized 16 nuclear SSR loci in *Phoenix dactylifera*. Most of these SSR markers amplified across 11 other *Phoenix* spp., as well as *Elaeis guineensis*, and 17 other palm taxa. These were the first SSR markers to be published for date palm. In Tunisia, Zehdi et al. (2002) used ISSR markers to examine phylogenetic relationships in Tunisian date palm accessions. Overall, considerable genetic diversity was observed, with results comparable to those of Trifi et al. (2000) using RAPD. However, the ISSR markers indicated that introductions from outside Tunisia, as well as male accessions, clustered within the main Tunisian cultivars. SSR markers have been utilized in other instances to characterize date palm genotypes in various other countries, including Oman (Al-Ruqaishi et al. 2008), Qatar (Ahmed and Al-Qaradawi 2009, 2010), and Saudi Arabia (Adbulla and Gamal 2010). Ahmed and Al-Qaradawi (2010) also utilized ISSR markers in their analyses. The work of Elshibli (2009) with date palm germplasm in Sudan is particularly interesting. Date cultivars from northern Sudan

were found to be diverse in regards to fruit morphology and chemical composition as well as SSR polymorphisms (Elshibli and Korpelainen 2009).

The date palm was thought to have a relatively small genome of approximately 250 Mb with genes distributed throughout approximately 41 % of the total genome, the remainder being non-coding regions; this is a wider distribution than found in other monocots (Barakat et al. 1999). However, recent work suggests that it is 550–650 Mbp in size (Malek 2010). Sakka et al. (2000) provided an early report of the construction of a DNA library from date palm. Little work has apparently been done on the chloroplastic or mitochondrial genomes of date palm. Benslimane et al. (1994) identified and characterized two minicircular plasmid-like DNAs from the date palm mitochondria: plasmid U (1,160 bp) and plasmid R (1,346 bp). These showed some homology to each other but not to nuclear, chloroplastic, or mitochondrial date palm genomes, nor other higher plant plasmid-like DNAs. An additional plasmid-like mitochondrial DNA was reported by Benslimane et al. (1996). This plasmid-like DNA (the plasmid S) was 98.8 % homologous with plasmid R. The R and S plasmids were never present in the same mitochondria and the pattern of their presence was related to bayoud resistance (Ouenzar et al. 2001) (see previous section). Analysis of progenies from controlled crosses suggested that the date palm mitochondrial genome was transmitted strictly maternally (Ould Mohamed Salem et al. 2007).

6.5.6 Hevea Rubber

Hevea brasiliensis, is exclusively cultivated over 11.23 million ha in the world for providing the industry with natural rubber (9.7 million mt in 2009). Natural rubber, 1,4 cis-polyisoprene is a renewable *green* elastomer being used mainly in the tire sector (70 %), in latex products (12), and in many other industrial applications.

Natural rubber is produced in Thailand (3,348,897 mt in 2011–29.6 % of the world total), Indonesia, Malaysia, India, Vietnam and also Ivory Coast, China, Sri Lanka, Brazil, Philippines, Liberia, Cambodia, Nigeria, Cameroon, Guatemala, Myanmar, Ghana, D.R. of Congo, Gabon, and Papua New Guinea. Rubber is currently planted in the form of grafted trees, at a density of about 450 trees per ha. The buds are collected from budwood grown in the budwood gardens, which are developed for the recommended clones. The plants produced in the nurseries can be budded stumps grown in the soil, or budded plants grown in plastic bags. Rootstocks can be also grown directly in the plantation field at standard density, with budding carried out at field level. Rubber tree experiences an immature phase that may vary from 5 to 9 years, depending on climate, soil conditions and management. When the trunk girth of the trees reaches 50 cm, tapping is initiated that may last between 15 and 30 years. The tapping, a periodically renewed cut incised in the bark of the trunk, generates latex (cell cytoplasm containing rubber particles) throughout the year (Jacob et al. 1995).

Application of molecular tools in rubber tree improvement has lagged behind because of limited knowledge of the genome. The genetic base of the cultivated rubber tree, *Hevea brasiliensis*, is assumed to be narrow. Molecular markers could be highly beneficial as a tool in assisting genetic characterization and breeding (Brondani et al. 1998; Nodari et al. 1997). Initially, hybridization-based RFLP markers, providing co-dominant information were used to characterize *Hevea* germplasm. RFLP technique was proved to be useful for genetic diversity study in wild and cultivated *Hevea* accessions using low copy number nuclear probes (Besse et al. 1994). RFLP analysis of organelle genomes of *Hevea* was also performed for establishing evolutionary relationships as these two genomes could reflect true evolution because of their uniparental inheritance (Luo et al. 1995). Using RAPD analysis Varghese et al. (1998) analyzed 24 cultivated *Hevea brasiliensis* clones to estimate genetic distance. Subsequently, Venkatachalam et al. (2002) described the genetic relationships for 37 *Hevea* clones using RAPD markers and the clones were classified into 7 major groups based on DNA markers. Venkatachalam et al. (2004) identified a dwarf specific RAPD marker and studied inheritance pattern among F1 hybrid progenies. Mathew et al. (2005) studied the phylogenetic relationship among three species of rubber, *H. brasiliensis*, *H. benthamiana* and *H. spruceana*, employing different molecular marker techniques namely, RAPD, chloroplast DNA PCR-RFLP and heterologous chloroplast microsatellites. RAPD analysis clearly indicated a high degree of polymorphisms among the three species. DNA fingerprints in *H. brasiliensis* using heterologous minisatellite probes from humans were reported by Besse et al. (1993). Low et al. (1996), for the first time, detected microsatellites in the *Hevea* genome through the database search of some *Hevea* gene sequences. The construction of a microsatellite-enriched library in *H. brasiliensis* was reported by Atan et al. (1996).

Genetic linkage map presents the linear order of markers (genes and other identifiable DNA sequences) in their respective linkage groups depicting the relative chromosomal locations of DNA markers by their patterns of inheritance. The linkage map allows revelation of more and more restricted segments of the genome and undoubtedly enhances our understanding in many areas of plant systematics. A genetic map for *Hevea* spp. was constructed using a population derived from an interspecific cross between PB260 (*H. brasiliensis*) and RO38 an interspecific hybrid clone (*H. brasiliensis* × *H. benthamiana*) following the pseudo-testcross strategy (Lespinasse et al. 2000a). The markers were assembled into 18 linkage groups, thus reflecting the basic chromosome number, and covered a total distance of 2,144 cM. A total of 717 loci constituted the synthetic map, including 301 restriction fragment length polymorphisms, 388 amplified fragment length polymorphisms, 18 microsatellites and 10 isoenzymes. Homologous linkage groups between the two parental maps were merged using bridge loci. Average marker density was 1 per 3 cM. Lespinasse et al. (2000b) mapped Quantitative trait loci (QTLs) for resistance to South American leaf blight (SALB), a disease of the rubber tree caused by the fungus *Microcyclus ulei* using the same cross combination (PB 260, a susceptible clone and RO 38, a SALB resistant clone). Eight QTLs for resistance were identified on the RO38 map, whereas only one QTL was detected on the PB260 map.

Isoprenoid biosynthesis is brought about through the mevalonate dependant metabolic pathway (Hepper and Audley 1969). Although it is known that biosynthesis of natural rubber takes place by a mevolonate pathway molecular biological characterization of related genes has not been adequate. Initial understanding on the regulation of gene expression in the laticifers of *Hevea brasiliensis* came from the study of Kush et al. (1990) who demonstrated for the first time that transcript levels of genes involved in rubber biosynthesis and genes induced by wounding and ethylene treatment were higher in laticifers than in leaves. Rubber particle in the laticifers is the site of rubber (cis-1-4-polyisoprene) biosynthesis. A 14 kDa protein, rubber elongation factor (REF), is associated with the rubber particle. To obtain more information concerning the function of REF and its synthesis and assembly in the rubber particle, Goyvaerts et al. (1991) isolated cDNA clones encoding REF and characterized the same. Biosynthesis of natural rubber is known to take place biochemically by a mevalonate pathway including six steps catalyzed by corresponding enzymes (Sando et al. 2008). A key enzyme involved in rubber biosynthesis is HMG-CoA synthase, which catalyses the condensation of acetyl-CoA with acetoacetyl CoA to form HMG-CoA (Sirinupong et al. 2005; Suwanmanee et al. 2002, 2004). Reduction of HMG-CoA to mevalonic acid, catalyzed by HMG-CoA reductase is considered as a rate-limiting factor in rubber biosynthesis and thereby regulating the biosynthesis of natural rubber. These two enzymes possibly function in concert in response to the supply of substrates for rubber biosynthesis (Suwanmaneea et al. 2002). HMGS mRNA transcript accumulation was found to be more in laticifers than in leaves. A positive correlation was also observed between the activity of HMGS and dry rubber content of the latex. Two members of HMGS from *H. brasiliensis* hmgs-1 and hmgs-2 were cloned and characterized. hmgs-I was found to be higher in laticiferous cells than in leaves whereas the abundance of hmgs-2 was more in laticifer and petiole than in leaves. In the case of *HMGR*, three genes hmg-1, hmg-2 and hmg-3 were identified of which hmg-1 was reported to be involved in rubber biosynthesis (Chye et al. 1991, 1992).

Defense/stress related genes namely MnSOD, HEVER, hevein, chitinase, β-1,3-glucanase are expressed in laticifers of *Hevea*. Miao and Gaynor (1993) isolated Mn-SOD, which was found to express in all tissues i.e., leaf, petiole, root, latex and callus and highest level expression was noticed in young leaves through northern analysis. A novel stress-induced gene, HEVER (*Hevea* ethylene-responsive) from the rubber tree was isolated and characterized (Sivasubramaniam et al. 1995). A multigene family encodes HEVER. HEVER transcript and protein were induced by stress treatment with salicylic acid and ethephon. β-1,3- glucanase gene was identified from a cDNA library derived from latex by Chye and Cheung (1995) and its higher expression level was noticed in latex compared to leaf. Thanseem et al. (2003, 2005) also cloned and characterized the same gene from Indian *Hevea* clones and demonstrated prolonged accumulation of β-1,3-glucanase transcripts in abnormal leaf fall tolerant RRII 105. Hevein, a lectin-like protein, belonging to a multigene family was found to play a crucial role in the protection of wound sites from fungal attack through latex coagulation (Broekaert et al. 1990; Pujade-Renaud et al. 2005). Over expression of chitinase was noticed during fungal infection and by

ethylene stimulation. In addition to the role in defense responses, expression of hevein and chitinase is linked to the characteristics of the latex flow. The products of hevein (procoagulant) and chitinase (anti-coagulant) genes, which compete for the same site (the N-acetyl-glucosamine moiety) of the hevein receptor to induce or inhibit the process of coagulation could be used as molecular markers for assessing yield potential of rubber clones. Such markers are of help in early selection of high yielding and stimulation responsive rubber clones (Chrestin et al. 1997). The full-length cDNA encoding a cysteine protease, designated HbCP1, was isolated for the first time from *Hevea brasiliensis* (Peng et al. 2008). The predicted HbCP1 protein possessed a putative repeat in toxin (RTX) domain at the N-terminal and a granulin (GRAN) domain at the C-terminal. In plants, cysteine protease are involved in diverse physiological and developmental processes including biotic and abiotic stresses. Transcription pattern analysis revealed that HbCP1 had high transcription in laticifer, and low transcription in bark and leaf.

The past several years have witnessed major advances in our understanding of plant genomes and genomic information through whole genome sequencing. The increasing availability of data from several plant genome-sequencing projects provides a promising direction for investigating genes and their functional and sequence homologs involved in plant development (Avraham et al. 2008). Although genome-sequencing projects lead to the identification of the complete catalogue of genes of an organism, they do not consider the gene expression patterns. Large-scale end sequencing of cDNA library generates ESTs, representing genes expressed in particular tissues or under particular developmental or environmental conditions. They have also been the target of sequencing in many of the projects and found invaluable for genome assembly and annotation. Whole genome sequence information helps in many aspects of plant trait improvement through gene discovery to transgenesis and use of molecular markers in breeding. *Hevea* genome sequencing project has already been launched jointly by Tun Abdul Razak Research Centre (TARRC) of the Malaysian Rubber Board in UK and newly established Genome Analysis Centre at Norwich, UK, and probably RRIM 900 series clone will be sequenced and draft sequence has been made available (Rahman et al. 2013). Very recently IRRDB also proposed to do whole genome sequencing of rubber in Biotechnology meeting at CIRAD in France. Quantum of *Hevea* genome sequencing work is a monumental task as the haploid genome size is enormous ($\sim 4 \times 10^3$ Mbp as per our calculation based on the DNA content measured by Leitch et al. (1998) and also rubber possesses a high-complexity genome with >60 % repetitive sequences.

Transgenesis is referred to the introduction of heterologous or homologous DNA into plant genome resulting in its stable integration and expression. The technology has played a critical role in defining the in vivo functions of plant genes. In recent years, with the rapid increase in gene sequence information, systematic transgenic approaches have been taken to characterize large number of genes in both reverse and forward genetic studies. As one of the experimental methods in functional genomics, transgenesis has the advantage of revealing the direct link between gene sequence and function; such results not only provide further the understanding of basic biologic question but also facilitate exploitation of genomic information for

crop improvement (Dixon et al. 2007). There are many variations of gene transfer methods to introduce transgenes into the plant genome. Genetic transformation offers a potential tool to breeders for introducing valuable traits to crop plants leading to the development of elite clones in a relatively short period of time. The most widely used methods are *Agrobacterium*-mediated gene transfer and biolistic transformation.

Conventional rubber breeding takes more than 25 years to develop a new clone (Priyadarshan and Clément-Demange 2004). The first transformation report in *Hevea brasiliensis* was published by Arokiaraj and Wan (1991) through *Agrobacterium*-mediated transformation. The first transgenic *Hevea* plants, using anther-derived calli as the explant of the clone Gl1 was successfully developed by Arokiaraj et al. (1994) following biolistic transformation method. Subsequently, a transgenic plant was developed using *Agrobacterium-mediated* gene transfer of anther-derived calli (Arokiaraj et al. 1996, 1998). Transformation efficiency could significantly be enhanced when the friable callus treated with calcium chloride and cultured on calcium-free medium prior transformation (Montoro et al. 2000). Inner integument tissue of the immature fruit of the clone PB260 was used as the explant for genetic transformation (Montoro et al. 2003). Transgenic plants of *H. brasiliensis* PB260 were developed through *Agrobacterium*-mediated transformation by Blanc et al. (2005). It was further reported that anther-derived embryogenic callus was the most suitable explant for genetic transformation (Rekha et al. 2006). Earlier transformation events were only with various marker genes. Later the experiments were focused on transferring various agronomically important genes into *Hevea* with enhanced tolerance to abiotic stresses, production of recombinant proteins etc. Subsequently, attempts were made to increase the SOD enzyme activity by overexpression of the same genes in *Hevea*. Transgenic plants were developed with SOD gene under the control of CaMV 35S and FMV 34S promoters (Jayashree et al. 2003; Sobha et al. 2003a). Biochemical analysis of the transgenic embryogenic calli of *Hevea* with SOD indicated significant increase in the activity of superoxide dismutase, catalase and peroxidase as compared to the control (Sobha et al. 2003a, b). Jayasree et al. (2003) reported successful development and establishment of transgenic rubber plant with SOD gene for their further evaluation. Genetic transformation experiment to over-express hmgr1 gene, involved in latex biosynthesis, in *Hevea* was performed by Arokiaraj et al. (1995). They generated transgenic embryos, which failed to produce any transgenic plant. However, they showed enhanced hmgr activity in the transformed calli.

Experiments were also undertaken for the production of foreign proteins in the latex of *Hevea*. The Pará rubber tree, which produces enormous volume of latex upon tapping could easily be exploited without any destruction for the production of foreign proteins in the latex throughout the year. Recombinant protein may be expressed in the specific parts of the plants or in specific organelle within the plant cell using tissue-specific promoters. Human serum albumin protein was expressed in transgenic *Hevea* plants by Arokiaraj et al. (2002). To characterize tissue-specific promoters derived from latex biosynthesis genes, transgenic approaches were adopted by Priya et al. (2006). They cloned and characterized promoter sequence of

the rubber elongation factor gene. A significant achievement towards antibiotic marker-free *Hevea* transgenic development avoiding the constraints of GMO regulations was made by Leclercq et al. (2010). They developed an efficient genetic transformation procedure in the clone PB260 using a recombinant green fluorescent protein (GFP). They showed GFP selection is less time consuming in terms of callus sub-culturing and offered the possibility of producing antibiotic resistant marker free transgenic plant.

Transcriptome sequencing and development of microarrays have been undertaken recently in *Hevea* rubber (Salgado et al. 2014; Triwitayakorn et al. 2011). Sequencing of transcriptomes of bark that leads to EST-SSR markers is also of prime importance (Cubry et al. 2014; Li et al. 2012) that calls for rigorous research. Such developments are certainly welcome that elevates *Hevea* rubber research on par with other tropical tree crops. However, such innovations must help to find answers to intriguing issues like Tapping Panel Dryness (TPD), stock-scion interactions and yield differences exhibited among trees raised through bud-grafting, molecular markers for selecting of high yielders at juvenile stage, delineation of parents of open pollinated seedlings, production of natural somatic seeds and so on.

6.6 Conclusions and Prospects

Breeding vis-à-vis genomics of tropical tree crops has been succinctly explained in this review. Tree crops are no way near to annual crops in genomic research. This lacuna is due to the fact that tropical species grow mostly in developing countries that are constrained with lack of enough funds to do advanced research. While the latest technology like transcriptional profiling is being used increasingly in annual crops, such techniques are to yet to make a take off in tree species. On the other hand, the conventional breeding of trees had immense opportunities that catered to the needs of human kind. Conventional breeding and molecular biological tools are expected to work in tandem. However, the progress in molecular biology of trees is meager. Factually, this is due to the recalcitrance of trees and the slow results coming to a researcher that can fully substantiate as a back up for the conventional breeding. Except in poplar trees, gene sequencing has not been seriously attempted in other tree species/crops. White spruce (Birol et al. 2013), mulberry (He et al. 2013) and *Hevea* rubber (Rahman et al. 2013) are in the offing with draft genome sequences, expected to make inroads for pathogen resistance, wood quality, growth rates and adaptation to changing climate. Cocoa, papaya, oil palm and a number of other fruit trees are ahead in the race (Schnell and Priyadarshan 2012). This is welcome, but the progress is inadequate to support or work in tandem with the conventional breeding strategies. The future of genomics of trees is bright but only serious investigations can help to accelerate the *systems breeding* that integrates information on gene function, genome states and regulatory networks across populations

and species that contributes to genetic and epigenetic variation to phenotypes and field performance.

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Chapter 7
Coconut Breeding in India

Raman V. Nair, B.A. Jerard, and Regi J. Thomas

Abstract *Cocos nucifera* L. is a perennial multipurpose palm grown widely in the humid tropics. It provides nutritious food and refreshing drink, edible oil and non-edible uses, fiber of commercial value, shells for fuel and industrial uses, timber and a variety of miscellaneous products for domestic and industrial uses. It is grown mainly in coastal areas and island ecosystems sustaining the livelihood of people and protecting the environment. There are conflicting theories regarding the origin and domestication of coconut. Coconut populations worldwide have been classified into two major groups: the Pacific group with five sub-groups (Southeast Asia, Melanesia, Micronesia, Polynesia and the Pacific coast of Central and South America), and the Indo-Atlantic group. The genetic resources in coconut are widely exploited through selection, hybridization for a number of desirable traits and have resulted in the development of many varieties. Breeding efforts are mostly confined to conventional approaches such as mass selection and hybridization, besides attempts to use individual palm selection for novel traits. Its perennial nature, heterozygosity, long juvenile phase and lack of technologies for mass propagation of palms with targeted traits are the challenges in breeding efforts. This chapter covers conventional breeding approaches such as mass selection and hybridization, information on varietal groups, identified genetic resources, breeding methods and techniques, current status and future strategies of coconut breeding in India.

Keywords Breeding • Coconut • *Cocos nucifera* • Genetic resources • Hybrid vigour • Improvement

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7.1 Introduction

Cocos nucifera L. is a member of the monocotyledon family Arecaceae (Palmae) in the subfamily Cocoideae that includes 27 genera and 600 species and is the only species of the genus *Cocos* (Perera et al. 2009). Coconut possesses a diploid genome with 32 chromosomes (2n = 2x = 32). It is an important multipurpose palm grown widely in the humid tropics and is referred to as Kalpavriksha (tree of life) in India considering that it provides nearly all necessities of life. Coconut provides nutritious food and refreshing drink, oil for edible and non-edible uses, fiber of commercial value, shells for fuel and industrial uses, timber and a variety of miscellaneous products for domestic and industrial use. In recent years, coconut is increasingly being considered as a health food, with virgin coconut oil, tender coconut water and inflorescence sap being promoted for consumption. There are conflicting theories regarding the origin and domestication of coconut. A number of theories supported a New World origin of coconut (Cook 1910) with subsequent dispersal to Asia and Polynesia. However, the theory for Polynesian and Asian origin has been postulated and convincing evidence has been provided for a Southeast Asian origin and Indo-Pacific domestication (Harries 1990).

However, at present, the origin of coconut is still not fully resolved with reports that wild specimens have been found growing in natural coastal forests in the Philippines and Australia (Greuzo and Harries 1984) supporting the theory that coconut originated in the Western Pacific (Beccari 1963; Corner 1966; Moore 1973). A possible region for coconut domestication is Melanesia, on the coasts and islands between Southeast Asia and the Western Pacific, approximately between New Guinea and Fiji, but more recently a submerged continental region of Southeast Asia (Malesia) has been suggested (Harries 1990). Harries (1978) describes a possible evolutionary process for coconut based on natural selection and evolution of large-fruited coconuts from a small-fruited progenitor. Studies suggest that dwarf coconuts may have originated as a result of inbreeding among tall coconuts as they show limited self-pollination. Purseglove (1968) states that dwarfs are probably mutations of tall types.

The perennial nature of coconut palms, a high level of heterozygosity, a long juvenile phase, and the need for large land areas and a long time for experimentation, along with the lack of technologies for mass propagation of coconuts with targeted traits are factors impeding successful breeding efforts. Breeding efforts are mostly limited to conventional approaches such as mass selection and hybridization, besides attempts to use individual palm selection for novel traits. Recent developments in biotechnological tools have resulted in considerable advancements in the identification of parental lines, elucidation of diversity, molecular characterization and hybridity testing (Rajesh et al. 2012).

This chapter covers information on varietal groups, identified genetic resources, breeding methods and techniques, efforts made for varietal development, and the current status and future strategies for coconut breeding in India.

7.2 Varietal Groups

Coconut classification is not standardized, resulting in the use of different terminologies to describe different coconut types by researchers. However, the major classification of coconut is based on stature and breeding behavior, dividing them broadly into two groups or types: tall and dwarf (Narayana and John 1949). The tall type is primarily outcrossing while the dwarf type is mainly self-pollinating (with a few exceptions). The tall cultivars are most common for commercial production in all coconut growing regions worldwide while the dwarf varieties are usually grown for ornamental and breeding purposes. The varieties currently grown in different coconut growing regions are mostly selections from the tall or dwarf cultivars or hybrids between the two. The present level of genetic diversity in coconut populations are the result of natural evolution and adaptation, as well as human intervention in the exploitation of the species.

One system of classification, based on the observation of several morphological traits, classified the known cultivars of coconut into var. *typica* (talls), var. *nana* (dwarfs) and var. *javanica* (intermediate) with two mutant varieties var. *androgena* (male coconut palm) and var. *spicata* (unbranched inflorescence). On the other hand, based mainly on the fruit characters and seed-germination traits, the terms *Niu Kafa* and *Niu Vai* are used to represent wild and domesticated coconuts, respectively. Based on the results of analysis using molecular markers, coconut populations have been classified into two major groups: the Pacific group with five sub-groups (Southeast Asia, Melanesia, Micronesia, Polynesia and the Pacific coast of Central and South America) and the Indo-Atlantic group. The Pacific group includes the domesticated coconut while the Indo-Atlantic group, includes Niu Kafa coconut types. However, no single method of classification can account for the variability observed in the coconut populations worldwide (Perera et al. 2009).

The morphological variation patterns observed worldwide in coconut populations are possibly attributed to the following factors, as enumerated by Harries (1978) and Lebrun et al. (1999):

(a) Natural selection, for sea-borne seed dissemination between islands and colonization of isolated coastal locations;
(b) Direct and indirect selection during domestication, for fruit traits, biotic and abiotic stress;
(c) Introgressive hybridization when domestic forms were dispersed by humans to localities where the wild forms already existed;
(d) Genetic drift in natural and introduced populations.

Coconut collection, conservation and evaluation of genetic diversity is the primary step in breeding programs and the utilization is dependent upon the local adaptability of the selected types and usage of targeted traits. The diverse types among different tall and dwarf types are predominantly used in breeding programs targeting the breeding objectives. The major differences between tall and dwarf are summarized in Table 7.1.

Table 7.1 Morphological characteristics of tall and dwarf coconut types

Trait	Tall	Dwarf
Trunk circumference	Sturdy with bulbous base	Thin cylindrical
Initiation of flowering	Late (5–7 years)	Early (3–4 years)
Mode of pollination	Highly cross-pollinated	Highly self-pollinated
Color of fruits and petioles	Generally mixtures of greens and browns	Either pure green, yellow, red (orange) or brown
Height increment per year	Greater than 50 cm	Less than 50 cm
Nut size	Very large to very small	Very small to medium
Phenotypic variation		
Within cultivar	High	Low
Between cultivars	High	High
Root distribution	Generally more dense and plentiful	Less dense and few
Leaf and bunch attachment	Very strong	Fragile

Source: Niral et al. (2010)

Tall palms, referred as var. *typica*, are commonly cultivated commercially in all coconut growing regions worldwide. They are sturdy and may continue to bear fruits for 80–100 years. Tall cultivars occupy the major coconut area in coconut growing regions.

Dwarf palms are referred as var. *nana*. They have a short productive life of about 40–50 years. Though initially confined to homestead cultivation, dwarf cultivars are being cultivated now at a large-scale for tender nut purpose. Tender coconuts from dwarfs are preferred over talls, as they have high total soluble solids, low acidity, high total and reducing sugars, high potassium and tasty nut water. Some of the dwarf cultivars are also promoted as they possess resistance to phytoplasmal diseases such as lethal yellowing.

Both tall and dwarf types have been utilized for the development of hybrid varieties, combining the early-flowering trait of dwarfs with the hardiness and high-yielding character of tall parents, and also exploitation of hybrid vigor. Some of the important tall and dwarf coconut cultivars and distinct trait specific accessions are listed in Table 7.2.

7.3 Breeding Methods and Techniques

As the sole species of the genus *Cocos*, coconut breeding is limited to the intraspecific level utilizing the selected diversity for specific traits as per the breeding objectives. Use of traditional breeding methods in coconut is limited due to the long generation interval, high level of heterozygosity, lack of a reliable method for vegetative propagation and limited seed production capacity of palms under artificial pollination. Although attempts have been made by several researchers, obtaining a

Table 7.2 Important tall and dwarf coconut cultivars

Region	Tall cultivars	Dwarf cultivars
Southeast Asia	Malayan, Klapawangi, San Ramon, Laguna, Dalig, Makapuno, Bali, Tenga, Thai Tall	Aromatic green, Nias Yellow, Nias Green, Bali Yellow, Coco Nino, Catigan, Tacunan, Malayan (red, yellow, green) Dwarf
Central and South America, Atlantic	Surinam, Jamaica, Sanblas, Panama Tall	Surinam Brown, Brazilian Green, Malayan Dwarf
Africa	East African, West African Tall	Cameroon Red, Pemba Dwarf
Pacific Ocean Islands	Markham, New Guinea, Karkar, Rotuma, Fiji, Samoan, Rangiroa, Lifou, Solomon, Rennell, Vanuata, Gazelle Peninsula Tall	Niu Leka, Hari Papua, Madang Brown, Vanuatu Red, Malayan Dwarf
Indian Ocean Islands	Seychelles, Comoros, Coco Raisin, Coco Bleu, Sambava Tall	Pemba (red, yellow, green) Dwarf
South Asia	Indian West Coast, Indian East Coast, Tiptur, Andaman Giant, Andaman Ordinary, Lakshadweep Ordinary, Lakshadweep Micro, Kaithathali, Kappadam, Benaulim, Sri Lankan, Gonthembili	Chowghat (orange, yellow, green), Gangabondam Green Dwarf, Lakshadweep (yellow, green, orange), Andaman Dwarf (yellow, green, orange), King Coconut, Sri Lankan (red, yellow, green)

Source: Niral et al. (2010)

pure line from heterozygous coconut has still not been achieved because of the long vegetative phase. Thus, coconut breeding is confined to mass selection of phenotypically superior parental palms, and their use in inter-varietal hybridization. Mass selection is the basic method for coconut breeding. Traditional methods of mother palm selection followed by seedling selection continue to be widely practiced by coconut farmers and breeders (Jack 1930; Liyanage 1953).

7.3.1 Mother Palm Selection

Mother palm selection is the most widely practiced method for coconut improvement. The criteria for mother palm selection vary with cultivar, location, nature of the garden and purpose for selection. Generally, palms consistently yielding 80 or more nuts per year and with a copra yield of 20 kg/palm/year are considered as preferred mother palms. The mother palms should be at least 25 years old to ensure the continued performance of the parental palm. Studies have indicated that the average number of female flowers, number of functional leaves in the crown, total leaf production up to 3 years after sowing and time taken for flowering are important components showing the largest direct effect on yield and thereby indicating their value in selection program.

As a monoecious palm, coconut produces male and female flowers on the same palm. The coconut inflorescence is a spadix with a branched spike having a number of spikelets. Each spikelet bears numerous male flowers mostly on the terminal end and a few female flowers towards the proximal end. Understanding the floral biology in individual palms is very important for choosing a palm for breeding work since the male and female flowers come to maturity at different times. Generally, the male phase is followed by the female phase where the former is the time interval between the opening of the first male flower and shedding of the last male flower in an inflorescence. The female phase is the time interval between the receptive stage of the first opened female flower and the last opened female flower in an inflorescence. Generally, the male phase lasts 18–25 days from the day of inflorescence opening depending on the cultivar and the location of palms during which active pollen dispersal takes place. The female phase is always much shorter and lasts 3–9 days depending on the cultivar and location of palms. Generally, in most tall accessions, there is clear time gap between these two phases (i.e. no overlap) making cross-pollination the only option for fruit set. On the other hand, in most dwarf accessions, these phases overlap either within an inflorescence (intra-spadix overlapping) or between successive inflorescences (inter-spadix overlapping) or both types of overlapping making the possibility of self pollination more in these accessions. Some tall accessions and most hybrid palms exhibit inter-spadix overlapping of male and female phases which is useful in identifying hybrid palms among the mother palms. The expression of male and female phases must be considered before making the choice of mother palms for any crossing program as this would help in identifying the typical dwarf or tall palms in the population (Thomas et al. 2015).

7.3.2 Seedling Selection

Nursery selection of seedlings is based on characters which are correlated with high yield of adult palms, such as early sprouting, faster growth rate, early splitting of the unexpanded leaf into leaflets, seedling vigour in terms of girth at the collar, height and number of leaves and color of petioles. From pollination to seedling selection, 2 years are required; the first for seed nut production and the second for the seedling selection. Considering the fact that the selected parental palms are also expected to give variable hybrid progenies due to the inherent heterozygosity, seedling selection is to be followed among hybrid progenies to have a homogenous hybrid population.

Due to the inherent heterozygosity and absence of pure lines, the effectiveness of mass selection through open pollination is limited. Therefore, mother palms selected on the basis of the progeny testing for seedling characters have been advocated to give a more scientific thrust to varietal improvement. Molecular markers may be useful in identifying the progenies as suggested by Rajesh et al. (2013).

Table 7.3 Coconut varieties developed through selection

Variety	Selection from	Agency
Chandra Kalpa	Laccadive Ordinary	CPCRI
Pratap	Benaulim Green Round	KKV
Tender nut variety	Chowghat Orange Dwarf	CPCRI
VPM-3	Andaman Ordinary	TNAU
Kera Chandra	Philippines Ordinary	CPCRI
Kamrupa	Assam Tall	AAU
Aliyarnagar-1	Arasampatti Tall	TNAU
Kera Sagara	Seychelles	KAU
Kalparaksha	CCS-7	CPCRI
Kalpa Prathibha	IND 0168	CPCRI
Kalpa Mitra	IND 022S	CPCRI
Kalpa Dhenu	IND 006S	CPCRI
Kalpasree	IND 029S	CPCRI
Kalpatharu	IND 125S	CPCRI, TNAU
Kera Keralam	IND 069S	CPCRI, TNAU, BCKVV
Kera Bastar	IND 004S	DBSKKV, ANGRAU, IGKV
Kalyani Coconut-1	IND 031S	BCKVV
Gautami Ganga	IND 003S	ANGRAU

Source: Nair et al. 2010b

7.3.3 Evaluation of Cultivars

Evaluation of coconut genetic resources is useful in the identification of some trait-specific variability with reference to morphological traits, fruit component traits, ball copra production, drought tolerance and insect/disease resistance. Evaluation for yield has also been an important component of the coconut improvement program. Evaluation of local cultivars along with exotic cultivars in multi-locational trials has been a very successful method in coconut breeding and has resulted in the development of many new varieties at ICAR-CPCRI in India (Table 7.3; Fig. 7.1). The response of the selected germplasm provides information on the adaptive traits in the accessions and helps in identification of accessions for specific traits. The details of some distinct trait-specific accessions of coconut identified in India are shown in Table 7.4.

7.4 Breeding Objectives

Coconut breeding objectives are still primarily focused on high yield and weight of meat/copra that is important in terms of oil yield; improvement of these two traits is the priority in all coconut breeding programs. As the vegetative phase of

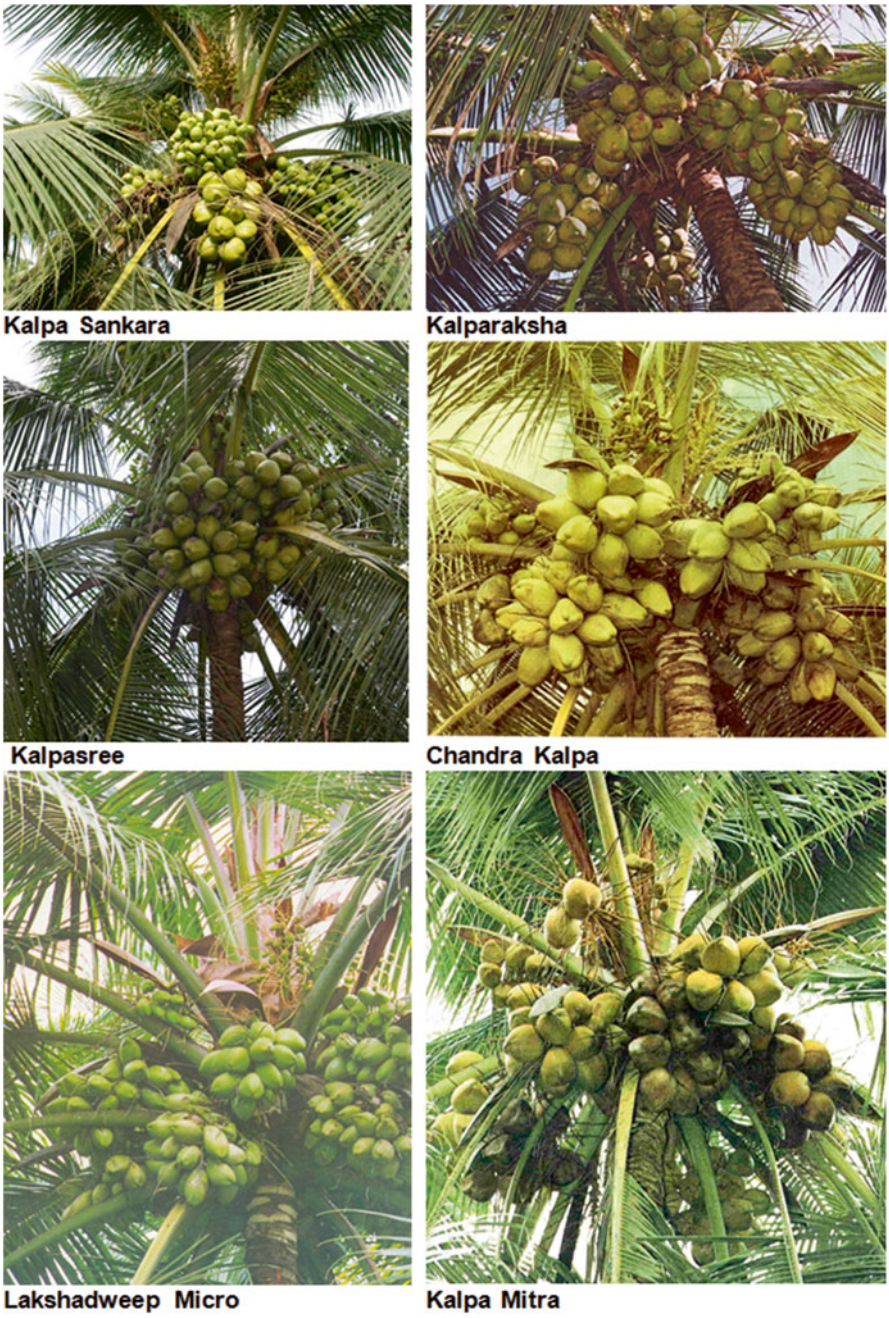

Fig. 7.1 Crown of some popular coconut varieties grown in India

Table 7.4 Some distinct trait-specific accessions of coconut

Trait	Accessions
High female flower production	Spicata Tall, Laccadive Micro Tall, Ayiramkachi Tall, Champin Micro Tall, Katchal Micro Tall
Large inflorescence	Borneo Tall
High copra content (>300 g)	San Ramon Tall, Malayan Tall, Markham Tall, Laccadive Giant Tall
Low copra content (<125 g)	Surinam Brown Dwarf, Chowghat Green Dwarf (CGD), Malayan Yellow Dwarf (MYD), Laccadive Micro Tall, Ayiramkachi Tall
High Oil content (>72 %)	Laccadive Micro Tall
Dwarf with high copra content (>200 g)	Camaroon Red Dwarf, Niu Leka Green Dwarf
High copra/oil output (>4 mt copra/ha and >2.5 mt oil/ha)	Fiji Longtongwan Tall, Adirampatnam Tall, Cochin China Tall, Java Tall, Philippines Ordinary
Ball-shaped copra production	Laccadive Micro Tall, Tiptur Tall, West Coast Tall (WCT), Ayiramkachi Tall, Java Tall
Good quality tender nut water	Chowghat Orange Dwarf (COD), Malayan Orange Dwarf (MOD), Philippines Ordinary Tall, Malayan Green Dwarf (MGD), Gangabondam Green Dwarf (GBGD), Cochin China Tall, MYD, Kulasekharam Green Tall (KGT)
Drought tolerance	Andaman Giant Tall, WCT, Java Tall, Federated Malay States, Laccadive Ordinary Tall, Cochin China Tall, Tiptur Tall
Root (wilt) disease resistance	Chowghat Green Dwarf (CGD), MGD
Eriophyid mite resistance	COD, KGT, WCT-spicata
Stem bleeding – less infection	Cochin China Tall, GBGD, Laccadive Ordinary Tall (LCT)
Aroma in coconut water and meat	Klapawangi Tall
Sweet endosperm	Mohacho Narel
Soft husk	Kaitathali Tall
Soft endosperm	Thairu Thengai or Nei Thengai or Ghee Thengai
Pink husk (tender fruits)	Guelle Rose Tall, West Coast Pink Tall

Source: Niral et al. (2010)

commercially-grown tall coconut varieties is long, taking over 5–7 years, precocity in flowering remains an important objective in coconut breeding. Significant progress has been achieved in precocity by combining early flowering behavior of dwarfs with commercially-grown tall coconut cultivars. The present focus of coconut breeders is to incorporate the tender fruit traits of dwarfs into the hybrids, along with the dwarfness and early flowering.

Breeding for oil content or for oil rich in a particular fatty acid is in the early phase as an objective in coconut breeding. With global climate change, droughts have become frequent in many coconut-growing countries and hence they constrain coconut production. Therefore, in the recent past, breeding for drought tolerance

has become a goal in the programs of many coconut growing countries. Selection of high-performing cultivars and individual genotypes in fields prone to droughts has been given top priority with the objective of selecting parents for breeding programs or improvement of local varieties through selection.

Although efficient and effective chemical and biological control methods have been developed against major and minor coconut pests of coconut, emerging and invasive pests owing to climate change and other factors pose a major threat to major coconut populations. As an example, most control measures that were field tested so far under experimental conditions have either been unsuccessful or impractical to adopt to control the coconut mite; (*Aceria guerreronis*) a microscopic coconut pest that lives beneath the perianth of the nut causing damage to developing nuts (Sathiamma et al. 1998). Hence, breeding for tolerance to coconut mite is a current breeding goal in the coconut breeding program in India. Screening of coconut varieties for tolerance to coconut mite resulted in identification of some accessions such as Kenthali (Ramaraju et al. 2000), Chowghat Orange Dwarf (Nair 2000), and Kulasekharam Green Tall (Niral et al. 2014) with less incidence of mite. Tepal aestivation in female flowers, shape of the developing fruits, growth rate and pattern of fruit enlargement are some of the traits identified as contributing to a lesser mite incidence in coconut.

Similarly breeding for disease resistance has also been a current objective given the situation that root (wilt) disease of coconut drastically hinders production and productivity. Selections from Chowghat Green Dwarf and Malayan Green Dwarf populations were identified with a higher degree of disease resistance. Similarly, diseases such as lethal yellowing, weligama leaf wilt and cadang cadang disease have been reported to be a threat in different coconut growing countries, necessitating breeding for resistance against those diseases.

As coconut palms grow taller with age, trained manpower is required for harvesting and other operations. Considering the difficulties in getting such trained climbers, coconut farmers prefer to cultivate short-statured palms. Hence, breeding for short-statured palms is considered important as a current breeding objective to sustain coconut farming.

7.5 Breeding Efforts

Coconuts grown all over the world were mostly derived by mass selection from open-pollinated progenies or hybrids among the selections as explained above. The earliest selection of coconut reported dates back 8,000–14,000 years ago when coconut was selected and domesticated for large round fruits and rich in water as a source of sweet uncontaminated water for seafarers travelling from island to island. However, after commercialization of coconut during the nineteenth and twentieth centuries, the yield of copra per palm or one of its correlates has been the major criteria for selection of mother palms by farmers. The efficiency of mass selection of mother palms based on desirable characters has been studied extensively. Progeny

trials in early coconut breeding research have generated information for developing criteria for selection of mother palms. The progenies resulting from open-pollinated seeds are the basis of an improved population. Early studies also provided information on some useful correlates between seed characters, period to sprouting and flowering, and initial yield and copra output. Seeds that sprout early promote seedling height, leaf and root number leading to a shorter flowering period and higher production of copra (Vijaya Kumar et al. 1991). Coconut nursery management practices all over the world adopt this concept for culling weak seedlings from nursery beds. From the same progeny trials it was found that open pollinated progenies of certain coconut palms are uniformly high yielding giving a mean yield of about 35–40 % more copra than the population mean. That phenomenon was explained as possessing of sufficient dominant yield traits in those palms to pass on to their offspring despite having been indiscriminately pollinated by unknown palms. Such palms were described as prepotent palms though, their identification is laborious and time-consuming and relatively few prove to be prepotent from a large number tested, thus the quantity of seed nuts collected from them for further utilization is limited (Iyer et al. 1979). However, prepotency in coconut has not been clearly established through field experiments.

Raising the enormous quantity of improved seeds demanded by farmers either by artificial pollination or stringent mass selection is impossible, so seed gardens were designed specifically for mass production of improved coconut genotypes. Since improved genotypes are produced by controlled natural pollination, the concept of isolated seed gardens was followed for mass production of superior genotypes (Bavappa et al. 1986).

Selection and evaluation of promising accessions conserved both at the Central Plantation Crops Research Institute, India and the coordinating centers under the All India Coordinated Research Project on Palms and State Agricultural Universities have resulted in the development and release of 27 high-yielding varieties of coconut, suitable for India's different agroclimatic zones, through application of mass selection.

7.6 Exploitation of Hybrid Vigor

The discovery of hybrid vigor in coconut by Patel (1937), in a cross between West Coast Tall and Chowghat Green Dwarf made in 1932 at the Nileshwar Coconut Research Station, was a significant landmark in the history of coconut improvement. This important finding paved the way for the successful breeding programs in coconut not only in India but also in the Philippines, Indonesia, Sri Lanka, Ivory Coast and Jamaica. Superior varieties selected after evaluation of germplasm have been utilized in crosses to develop high-yielding hybrids.

Inter-varietal hybridization has shown the greatest gains in coconut breeding, demonstrating the usefulness of heterosis. Scientific coconut breeding began with the first controlled hybridization accomplished in Fiji in 1926 between Malayan

Red Dwarf and Niu Leka Dwarf (Marechal 1928). In India, the first hybridization between tall and dwarf (West Coast Tall x Chowghat Green Dwarf) was attempted in 1930, with the intention of combining the quality of copra from the tall parent and the high productivity as well as early flowering from the dwarf parent. Most of the hybrid tests that were conducted since then have involved dwarf x tall (inter-varietal) and tall x tall (intra-varietal) crosses and in these studies the superiority of hybrids over local tall cultivars was well established.

Although both types of hybrids are high yielding, D x T hybrids have distinct advantage over T x D hybrids as they could be produced on a large scale by regularly emasculating dwarf mother palms, permitting free natural crossing with the pollen of tall palms growing nearby. Therefore, in recent years, production of D x T hybrids rather than T x D hybrids has been advocated because of the ease with which they can be produced.

These hybrids are early-bearing and high-yielding in terms of copra and oil yield per palm. More than 100 cross combinations involving tall and dwarf accessions were evaluated at CPCRI and other centers and many high yielding hybrids have been released for cultivation in India.

Subsequently, T x T crosses between unrelated lines of tall varieties were also carried out to develop improved varieties with high output of good quality copra and tolerance to biotic and abiotic stresses. Individual trees of high breeding value were identified and these genotypes utilized for production of T x T hybrids. Although late-bearing, the yield potentials of T x T hybrids are good. In India, T x T hybrids have not yet been released for commercial cultivation. However, at CPCRI a few T x T hybrids are presently under evaluation for their drought-tolerant nature.

Strategies to produce superior hybrids also include selection of parents for crossing on the basis of combining ability tests. The experience in Ivory Coast in the production of the MAWA (Malayan Dwarf x West African Tall) hybrid points to the need for employing combining ability tests such as Line x Tester, diallel, for selection of parents for the crossing program for the production of superior D x T and T x T hybrids with more homogenous performance. In India, diallel analysis involving 16 parental lines indicated Gangabondam Green Dwarf (GBGD) and Laccadive Ordinary Tall (LCT) to be good general combiners and the combination LCT x GBGD as the combination with the best specific combining ability for copra and nut yield. After the first successful attempt, numerous hybrids have been produced by crossing different dwarf and tall cultivars originating from various geographical regions. The crosses COD x WCT, WCT x COD, LCT x COD, MYD x WCT, MYD x TPT, ECT (East Coast Tall) x MGD, ECT x MOD, ECT x MYD, GBGD x ECT, CGD x WCT are examples of some of the most promising present day coconut hybrids in India. In India, 19 hybrids, including 8 superior Dwarf x Tall hybrid varieties and 11 Tall x Dwarf hybrid varieties have been developed for commercial cultivation in different regions across the country (Table 7.5).

Although the first coconut hybrid tested in the world was a dwarf x dwarf hybrid, they are still the least exploited hybrids. Recently, attempts were made in India to evaluate dwarf x dwarf hybrids for identification of superior heterotic hybrids for tender nut yield and quality. Initial reports suggested the dwarf x dwarf hybrids as early flowering and they exhibit heterosis for growth and developmental traits.

Table 7.5 Coconut hybrids released for cultivation in India

Sl. no.	Hybrid	Parents involved	Agency
1	Chandra Sankara	COD X WCT	CPCRI
2	Chandra Laksha	LO X COD	CPCRI
3	Kera Sankara	WCT X COD	CPCRI
4	Laksha Ganga	LO X GB	KAU
5	Ananda Ganga	AO X GB	KAU
6	Kera Ganga	WCT X GB	KAU
7	Kera Sree	WCT X MYD	KAU
8	Kera Sowbhagya	WCT X SSA	KAU
9	VHC-1	ECT X MGD	TNAU
10	VHC-2	ECT X MYD	TNAU
11	VHC-3	ECT X MOD	TNAU
12	Godavari Ganga	ECT X GB	ANGRAU
13	Kalpa Sankara	CGD X WCT	CPCRI
14	Kalpa Samrudhi	MYD X WCT	CPCRI
15	Konkan Bhatye Coconut Hybrid-1	GBGD X ECT	DBSKVV
16	Kalpa Srestha	MYD X TT	CPCRI
17	Kalpa Ganga	GBGD X FJT	UHS
18	Vasista Ganga	GBGD X PHOT	Dr. YSRHU
19	Ananta Ganga	GBGD X LCT	Dr. YSRHU

Source: Niral et al. (2014)

Some dwarf dwarf combinations such as MYD x NLAD (Niu Leka Green Dwarf) produced a compact crown, long bunches with a high number of medium-sized fruits having higher kernel and tender nut water and sturdy stems with slow vertical growth; MYD x CGD with early flowering and bunch production; and COD x GBGD with early flowering and high yield in terms of number of fruits; these examples highlight the potential of D x D hybrids.

7.7 Breeding for Special Characteristics

7.7.1 Disease Resistance

Among diseases, root (wilt) is the most devastating to coconut in India. The characteristic symptom of the disease is flaccidity, yellowing and marginal necrosis of leaflets followed by a progressive decline in yield. Investigations carried out at CPCRI, Regional Station, Kayamkulam on the etiology of the disease, suggested the association of phytoplasma (Solomon et al. 1983). Considering the phytoplasmal etiology of the disease, development of resistant varieties is considered to be the practical solution for the management of the disease.

Screening of available coconut germplasm by planting seedlings in a disease-affected farm at CPCRI Regional Station, Kayamkulam was initiated in 1961. Radha (1961) reported a higher degree of resistance to both leaf rot and root (wilt)

in Andaman Ordinary Tall and New Guinea Tall based on disease incidence. Results of large-scale screening trials undertaken during 1972 at CPCRI, Kayamkulam and also in farmers' gardens revealed that all the cultivars and hybrids evaluated contracted the disease. In 1982, a trial involving 27 cultivars, 10 hybrid combinations, F_2 (OP) of D x T, and progenies of elite WCT palms was laid out in farmers' gardens. The results revealed that among the 27 cultivars all except Kenthali Orange Dwarf had contracted the disease (Jacob et al. 1998).

Based on the recommendations of the International Symposium on Coconut Research & Development, held at Kasaragod in 1976, Iyer et al. (1979) conducted a survey of natural populations in root (wilt) disease-affected areas in 1977–1981 to identify elite super palms exhibiting high-yielding potential. A total of 12 elite palms were selected from the root (wilt) affected areas of Kerala State. Open-pollinated seed nuts from these palms were raised and planted in root (wilt) disease affected areas. However, all of them became infected with the disease in subsequent years.

A comprehensive breeding program for evolving resistant/tolerant coconut varieties was implemented at CPCRI, Kayamkulam begining in 1988 (Nair et al. 2010a). The disease-free and high-yielding palms found in the midst of heavily-diseased palms and located in disease hotspots were identified as the base material for the breeding program. The selected disease-free palms were selfed/inter se mated since 1990 to produce two sets of first generation progenies (self and inter se). Observations recorded after 18–19 years of growth revealed that the disease incidence in inter se mated progenies was only 47 % compared to 63 % in selfed progenies. The inter se mated progenies recorded 35–40 % higher nut yield compared to selfed progenies (Thomas et al. 2014). Systematic evaluation trials at CPCRI, Kayamkulam for developing varieties with resistance/tolerance to root (wilt) disease has led to the release of three coconut varieties for the root (wilt) disease prevalent area.

A survey carried out in *hot spots* of root (wilt) disease revealed that 75 % of the observed Chowghat Green Dwarf (CGD) palms were disease-free indicating that it has a higher level of resistance to root (wilt) disease when compared to other varieties. Besides, observations from a screening trial involving ten varieties revealed that CGD had the highest level of resistance (Nair et al. 2004). Accordingly, CGD was identified as the source of resistance for the breeding program. Considering the high yield and low incidence of root (wilt) disease, the selection made from CGD was released under the name Kalpasree for cultivation in homesteads of the root (wilt) prevalent areas (Fig. 7.1).

Subsequent studies carried out in 2004 resulted in identification of another promising variety, Malayan Green Dwarf as resistant to root (wilt) disease (Nair et al. 2007). This observation was recorded from a seed production plot at the Coconut Development Board Farm at Neriamangalam, planted with five dwarf varieties of coconut, namely, Malayan Green Dwarf (MGD), Malayan Yellow Dwarf (MYD), Malayan Orange Dwarf (MOD), Chowghat Green Dwarf (CGD) and Chowghat Orange Dwarf (COD). The popular cultivated variety West Coast Tall (WCT) was

treated as the control. WCT showed 84 % disease incidence indicating the availability of sufficient inoculum for evaluation of resistance of the dwarf varieties. With regard to resistance, CGD showed maximum resistance with disease incidence of 19.9 % followed by MGD with 22.4 %. MGD gave the highest nut yield (89) followed by WCT (49). Considering the high yield and resistance to root (wilt) disease, this MGD selection was released under the name Kalparaksha for cultivation in the root (wilt) prevalent areas (Fig. 7.1).

Observations on CGD x WCT progenies, planted during 1991 indicated that 70 % of the hybrids became infected with the disease within 18 years of planting. Even though a majority of CGD x WCT hybrids were diseased, they gave a 10 year cumulative average yield of 84 nuts/palm/year indicating that this hybrid is tolerant to root (wilt) disease. Among the hybrids, disease-free hybrids gave an average yield of 107 nuts/palm/year, whereas the disease-acquired hybrids gave 72 nuts/palm/year (Nair et al. 2006). Considering the performance of CGD x WCT in the root (wilt) disease prevalent area it was released under the name Kalpa Sankara (Fig. 7.1). Nair et al. (2010a) did an exhaustive review on breeding for resistance to coconut root (wilt) disease.

7.7.2 Pest Resistance

Although coconut is attacked by many pests, a very few such as the eriophyid mite, rhinoceros beetles and red palm weevil cause major economic losses in most cultivation areas. Among them, the eriophyid mite is a serious pest in almost all growing regions. The habit of the pest on the plants provides selection indices for tolerance. Variability for mite tolerance in coconut were reported owing to the traits of fruit color, fruit shape and tepal aestivation in the flowers. However, a conclusive test to determine resistance is still elusive. Round and dark green fruits show better tolerance against the eriophyid mite than the elongated fruits and of other colors, as reported by Moore and Alexander (1987). The entry of mites depends on the tightness of tepals to the fruits at the early stages of fruit development. The smaller the gap between fruit and tepal, the less mite incidence. Greater tightness is achieved in round rather than elongated and angled fruits. In India, Kulasekharam Green Tall, a selection from a Kulasekharam coconut population which is a derivative of Malayan Green Dwarfs, was found to suffer less mite infestation. Besides, Chowghat Orange Dwarf was also identified with less mite damage (Nair 2000) and the *spicata* mutant has shown a fair level of resistance to the mite. Laccadive Micro was identified with high mite infestation.

Although all the coconut cultivars are prone to damage by rhinoceros beetles, the hybrids resultant from Chowghat Orange Dwarf as pollen parent was reported to be more susceptible (Nambiar 1988). Sosamma et al. (1988) identified Java Tall, Klapawangi Tall, Kenthali and Andaman Giant Tall as more tolerant to burrowing nematodes.

7.7.3 Drought Tolerance

Many portions of the major coconut growing areas in India and other coconut growing regions are periodically subjected to low rainfall and a long dry period resulting in poor yield. Under these circumstances, developing drought tolerant varieties/hybrids is of great importance to increase coconut production in drought affected areas. Drought tolerance in coconut depends on many phenotypic and physiological traits. A cultivar with more roots and a fine root density is less affected by drought, as reported by Cintra et al. (1993). Physiological traits such as leaf stomatal frequency, stomatal index, chlorophyll fluorescence, epicuticular wax content, activities of lipases and proteases are major parameters for the identification of drought tolerant coconut cultivars (Rajagopal et al. 1988; Repellin et al. 1994). Based on morphological and physiological studies, drought-tolerant cultivars have been identified at CPCRI, namely, Federated Malay States, Java Giant, Fiji, Laccadive Ordinary and Andaman Giant (Fig. 7.1). These varieties are being utilized in the breeding programs. The high yielding hybrids, that is, Laccadive Ordinary Tall x Chowghat Orange Dwarf and Malayan Yellow Dwarf x West Coast Tall have been found to be drought tolerant (Kasturi Bai et al. 2006; Rajagopal et al. 2005). Screening coconut germplasm and its evaluation in drought-prone areas for their suitability would take a very long time. The possibility of utilizing the available plantations in drought prone areas for identification of drought tolerant palms based on the phenotype and the physiological parameters has been demonstrated (Naresh Kumar et al. 2006). Utilization of such in situ drought tolerant palms from drought affected areas in the breeding programs would be expected to reduce the time duration.

7.7.4 Cold Tolerance

As a tropical palm, coconut is highly susceptible to low temperatures. Flowering and fruit set is affected below 13 °C. Mao (1986), while investigating the meteorological indices for coconut cultivation in China, enumerated that the spear leaf damage, drying of leaves and uneven or wrinkled kernel inside the nuts are symptoms due to cold. Hainan Tall of China and Kamrup Tall of India are reported to possess cold tolerance (Chowdhury et al. 2001; Mao and Lai 1993).

7.7.5 Novel Traits

Some important spontaneous mutations with desirable traits have been reported in coconut which has potential to be used in breeding programs (Arunachalam et al. 2001). A spontaneous mutant *spicata* with many flowers on unbranched

inflorescence, the Thairu Thengai mutant with soft endosperm (Jerard et al. 2013a), the Kaithathali with an edible husk, sweet kernel coconut and flavored coconut are some of the novel mutants which have immense potential. Makapuno is a single recessive mutation, originally reported from the Philippines, where the endosperm becomes soft as butter. This mutant has been successfully exploited through controlled pollination among makapuno bearing palms followed by embryo-culture. Mutants similar to the makapuno type have been reported from other coconut growing regions, such as Coco Gra of Seychelles, Kopyor of Indonesia, Thairu Thengai or Nei Thengai or Ghee Thengai of India, Dikiri Pol of Sri Lanka, Mapharao Khati of Thailand, Sap of Vietnam, Niu Garuk of Papua New Guinea and Pia of Polynesia. Use of these soft endosperm mutants in the breeding of new varieties or developing pure populations would provide novel opportunities for product diversification in coconut.

The sweet kernel coconut (Mohacho Narel) is another important mutant form reported in India which has a sweet endosperm with very little or no fibers, making the kernel suitable as a salad for table purpose. Development of a large population bearing sweet kernel fruits is a challenging task as the inheritance and genetics need further evaluation and progeny testing (Niral et al. 2013).

Compact dwarf development is another important area in coconut breeding for which the extreme dwarf mutants from natural populations can be used, besides the identified prominent dwarf cultivars. One such extreme dwarf mutant identified in India (CPCRI 2010) from Lakshadweep coconut populations has shown extreme dwarfism compared to the conserved dwarf cultivars among the genetic resources. Use of such dwarf progenies would help in changing the plant type eventually making coconut suitable for high-density planting. The edible husk types and *spicata* are still under-exploited types although they have been collected long ago and conserved. Aroma in coconut is a novel trait which is released when a young coconut is cut open. The sweet flavor adds to the palatability in tender nut consumption. Klapawangi Tall and Aromatic Green Dwarf are cultivars available with this trait in India. However, a large population of these types with the desirable traits is still difficult to achieve owing to high heterozygosity that hinders production of true-to-type progenies.

Bulbil production is a rare phenomenon in coconut in which vegetative shoots are produced instead of an inflorescence, creating possibilities of getting clonal propagules in coconut (Mohandas et al. 1976; Sudasrip et al. 1978; Thomas 1961). Identification of bulbil-producing palms would be helpful in developing homogenous populations with vast potential in developing mapping populations and identification of markers for breeding, hitherto difficult with available heterogeneous populations (Jerard et al. 2013b). Finding out the factors for natural bulbil production and their inducement by artificial means in selected palms would be helpful in streamlining the precision breeding approaches in coconut. Suckering is another means of obtaining clonal propagules from coconut, which is also a rare occurrence in which one or more suckers are produced at the base of the palm.

The occurrence of natural haploids in coconut (Whitehead and Chapman 1962) is another important opportunity to breeders for developing dihaploids, which in

turn is helpful in strengthening breeding programs, as dihaploid parents are expected to give heterotic hybrids with a higher degree of uniformity than the normal diploids. With the advent of molecular biology and DNA assessment techniques, there is ample scope for screening large coconut nurseries for identification of natural haploids.

7.8 Embryo Culture and Tissue Culture

At present, embryo culture has become an important tool for safe germplasm exchange as it reduces transportation cost and meets phytosanitary regulations. CPCRI has standardized a simple protocol for culturing coconut embryos and this has been utilized in the overseas germplasm expeditions. The protocol includes direct field collection of 9–11 month old coconut embryos, short-term storage, in vitro retrieval and field establishment (Karun et al. 2008). The protocol for embryo culture has been successfully used for collection of both indigenous and exotic germplasm. It is also useful in producing plantlets from special coconut types such as Mohacha Narel and Thairu Thengai where the endosperm is soft and jelly-like and does not germinate under normal conditions.

Development of tissue-culture techniques aimed at rapid multiplication of elite planting material can greatly save time, space and resources. Coconut is among the recalcitrant species to regenerate in vitro. More than a dozen laboratories in India began working on tissue culture in the 1980s. Different explants such as the inflorescence, leaf and endosperm were used to induce callus. Plumular explants responds best to in vitro culture in coconut as compared to other explants. Success in coconut micropropagation has been reported for regeneration of complete plantlets from plumular tissues of coconut (Rajesh et al. 2005). Different stages of coconut plantlet production using plumular explants are depicted in Fig. 7.2a–h. This is being utilized for scaling-up the planting material production of released varieties of coconut, particularly the variety Kalparaksha which has been released for cultivation in the root (wilt) prevalent areas of Kerala, India.

7.9 Molecular Biology

DNA-based markers possess the potential to significantly increase the efficiency of coconut-breeding programs, especially in the areas of germplasm management, genotype identification and marker-assisted selection of economically-important traits. With the use of molecular marker technologies, a number of marker types, including inverse sequence tagged repeat (ISTR) (Rhode et al. 1995); restriction fragment length polymorphism (RFLP) (Lebrun et al. 1998); random amplified polymorphic DNA (RAPD) (Ashburner et al. 1997; Manimekalai and Nagarajan 2006; Upadhyay et al. 2004); amplified fragment length polymorphism (AFLP)

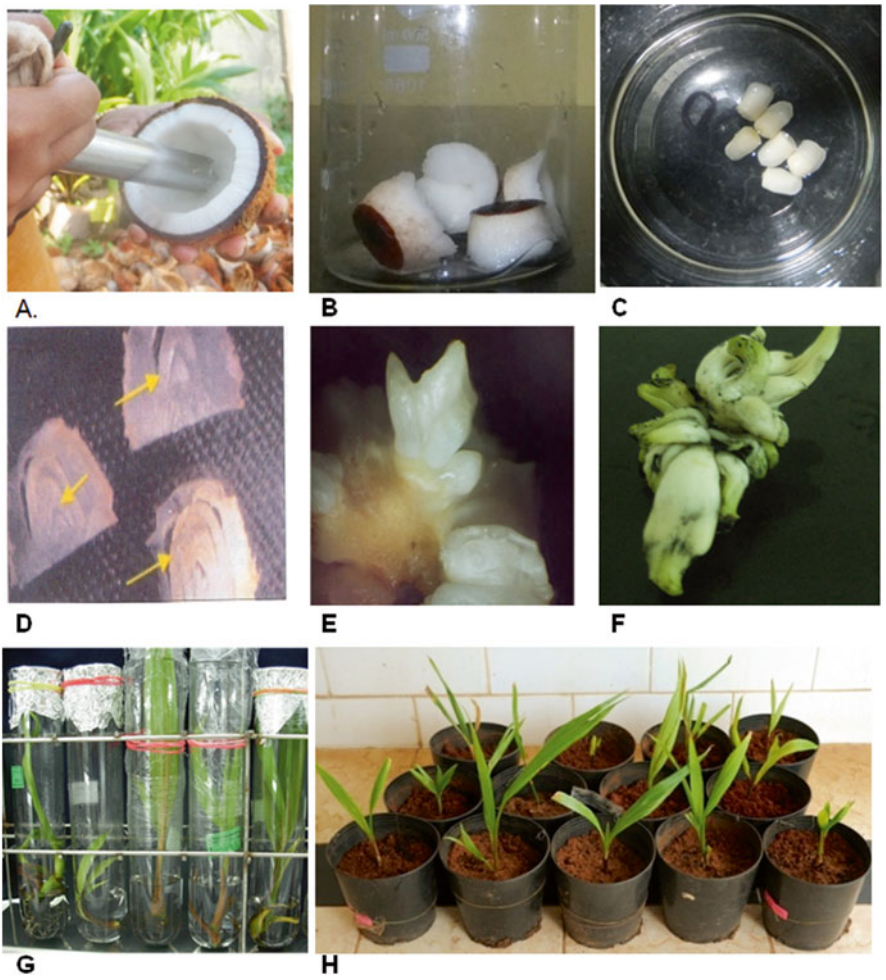

Fig. 7.2 Various stages of coconut plantlet production using plumule culture. (**a**) Scooping out embryo with cork borer, (**b**) Endosperm plugs, (**c**) Coconut embryo, (**d**) Plumular sections, (**e**) Somatic embryo germination stage, (**f**) Regeneration of plantlet, (**g**) Plantlet with shoot and root, (**h**) Plantlets ready for hardening

(Perera et al. 1998); AFLP and inter simple sequence repeat (ISSR) (Teulat et al. 2000); simple sequence repeat (SSR) (Merrow et al. 2003; Rivera et al. 1999); DNA amplification fingerprinting (DAF) (Jayadev et al. 2005; Nagaraju et al. 2003) and single nucleotide polymorphism (SNP) (Mauro-Herrera et al. 2007). These all have been used to study genetic diversity in coconut germplasm. Presently, work is being focused on characterization of conserved coconut germplasm through SSR analysis, identification of markers associated with economically-important traits as well as with dwarfness, and for selection of hybrid seedlings in the nursery. A SCAR marker has been identified as associated with the tall trait in coconut (Rajesh et al.

2013). Application of these molecular technologies in coconut improvement will help accelerate the breeding program through marker-assisted selection of superior genotypes at the juvenile phase, and also help in reducing the time and space required for experimentation.

Attempts were made to clone and characterize genes induced during somatic embryogenesis in coconut. SERK (somatic embryogenesis receptor kinase) genes play a key role during somatic embryogenesis in different crops. Sequence encoding SERK were cloned from coconut using a degenerate primer-based PCR approach. Sequence analysis revealed a high level of similarity to other plant SERKs. In addition, genes involved during somatic embryo formation such as SERK, MAP kinase and BABYBOOM have been cloned and characterized and gene-expression studies currently are in progress at CPCRI (Karun and Rajesh 2010).

7.10 Conclusions and Prospects

Although the large standard collection of coconut germplasm has been used for development of many varieties, there is an urgent need to utilize the special types such as aromatic coconut, sweet coconut and Thairu Thengai in breeding programs to diversify the coconut cultivation. Further selected varieties and hybrids produced by crosses between selected cultivars need to be tested in multi-location trials to assess the suitability of commercial cultivation in different agroecological zones of India. Considering the achievements made and opportunities available, the strategies suggested for future breeding programs include development of varieties for yield coupled with value-added products like inflorescence sap, developing stress-tolerant varieties to expand the coconut cultivation in non-traditional areas, development of inbred lines in coconut for production of vigorous hybrids, standardization of somatic embryogenesis protocol, application of molecular markers to aid in breeding programs, especially for biotic and abiotic stresses, and concentrated efforts for identification of hybrid seedlings in coconut nurseries.

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Part III
Abiotic Stress Tolerance

Chapter 8
Molecular Breeding to Improve Plant Resistance to Abiotic Stresses

Gundimeda J.N. Rao, Janga N. Reddy, Mukund Variar, and Anumalla Mahender

Abstract Tolerance to abiotic stress is an important agronomic trait in crops and is controlled by many genes/quantitative trait loci (QTLs). As abiotic stresses significantly affect grain yield, it is necessary to combat these stresses to minimize yield losses. The current status of the study of abiotic stress tolerance suggests the general role of some regulatory factors in the environmental adaptation mechanisms; therefore, it is also possible to find some common QTLs/genes influencing more than one type of stress at a time. Identification of these factors will not only contribute to the understanding of plant biology but also to achieve stable crop production around the world through breeding approaches. Recent functional genomics technologies have become the most useful tool for the understanding of tolerance to various abiotic stresses and the genetic enhancement of various crops is being carried out by through transfer of QTLs/genes using different approaches like marker assisted selection (MAS) genetic engineering. In view of the impressive progress in these areas in recent years, integration of biotechnological approaches like molecular breeding with conventional breeding should be the major emphasis to hasten the development of crops that are more tolerant to different abiotic stresses.

Keywords Abiotic stress • Abscisic acid • Cold • Drought • Ethylene • Freezing • Genetic engineering • Marker Assisted Selection (MAS) • Quantitative trait loci (QTLs) • Salinity

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8.1 Introduction

The world's food supply is obtained either directly or indirectly from plants, but fewer than 100 are used for food and about 50 species are actively cultivated (Burger 1981). The majority of crops such as wheat, barley, oats, rye, rice, maize, sorghum and millet provide 56 % of the food energy and 50 % of the protein consumed on earth (Stoskopf 1985). Cereals are the most important food crops and they continue to be the most important source of total food consumption in the developing countries (FAO 2006); the top three cereals are maize, rice and wheat and together they represent about 90 % of all cereals produced (http://faostat.fao.org/).

The global cereal production forecast of 2014 is nearly 2,480 million mt, down 1.4 % from 2013. While wheat production in 2014 is forecast at nearly 703 million mt, world rice production is predicted to reach about 503 million mt (milled basis) (FAO 2014) (http://www.fao.org/worldfoodsituation/csdb/en/). It has been estimated that land degradation, urban expansion and conversion of crops and croplands for non-food production will reduce the total global cropping area by 8–20 % by 2050 (Nellemann et al. 2009). Because over 80 % of total agriculture is rainfed, and water is vital to plant growth, any variation of precipitation patterns will have a significant impact on agriculture, and drought is a major environmental stress reducing crop yield around the world (Bruce et al. 2002). These facts pose a formidable challenge to increase food production by 50 % to meet the projected demand of the world's population by 2050.

Abiotic stresses such as drought, salinity, flood, high temperature and cold significantly constrain plant growth and metabolism that ultimately disturbs plant mechanism and reduces crop yield (Ahmad and Prasad 2012; Bray et al. 2000). Crop productivity is greatly affected by abiotic stress and a 50–70 % decline in major cereal crop production has been attributed to abiotic stresses (Mittler 2006). For example in rice, drought stress is the largest constraint to production in rainfed systems. In Asia, it affects 10 million ha of upland rice and over 13 million ha of rainfed lowland rice (Pandey and Bhandari 2007); the average yield reduction in rainfed, drought-prone areas has been found to be 17–40 % in severe drought years (Greenbio 2011). Rice is considered to be moderately sensitive to salinity (Gregorio et al. 1997) and soil salinity limits rice plant growth and development, resulting in yield losses of more than 50 % (Zeng and Shannon 2000); rice yields are reduced by 12 % for every unit of salinity (dS/ml) (Hanson et al. 1999; Maas and Grattan 1999). Flooding is a significant problem for rice farming and affects about 10–15 million ha of lowland rice fields in South and Southeast Asia and is causing an estimated USD 1 billion in yield losses per year. These losses could increase considerably given predicted sea level rise, as well as an increase in the frequency and intensity of flooding caused by extreme weather events (Bates et al. 2008). Higher growing season temperatures can significantly impact agricultural productivity, farm incomes and food security (Battisti and Naylor 2009). Similar trends in other cereals also suggest drastic reductions in both production and productivity due to abiotic stresses.

One of the most serious long-term challenges to achieve sustainable growth in cereal production is climate change (Adams et al. 1998; IFPRI 2001; Vaghefi et al. 2001; Wassmann and Dobermann 2007). Existing studies show that climate is the single most important determinant of agricultural productivity, basically through its effects on temperature and water regimes (Lal et al. 2005; Oram 1989). It is expected that by 2100, the earth's mean surface temperature is predicted to rise 1.4–5.8 °C and extreme events, such as floods, droughts and cyclones, are likely to become more frequent (IPCC 2007); droughts and floods are already causing widespread rice yield losses around the globe (IFAD 2009; IRRI 2010; Pandey and Bhandari 2007). In delta and coastal regions, climate change is expected to raise sea levels, and this will increase the risk of flooding and salinity problems in major rice-growing areas (Mackill et al. 2010; Wassmann et al. 2004). Rice yields will be severely affected by the increase in temperature of the earth due to the atmospheric concentration of carbon dioxide (Peng et al. 2004). Rice yield is found to be more sensitive to nighttime temperature: each 1 °C increase in nighttime temperature leads to a decline of about 10 % in rice yield (Peng et al. 2004; Welch et al. 2010). These predicted changes in climate are likely to further increase the economic vulnerability of poor rice producers, particularly in South Asia, where more than 30 % of the population is extremely poor (with income of less than USD 1.25 per day). Climate change presents an additional burden on the world's agricultural and natural resources, which are already coping with the growing food demand driven by population growth and higher income in developing countries (Wassmann et al. 2011).

This review explores the problems and recent developments in the area of modern biology in major cereals and the future impact on production. In this chapter, the abiotic stresses involved in major cereal crops, the advances made in the identification of genes/QTLs associated with abiotic stresses in each crop, the breeding attempts employing the marker-assisted approach and the results obtained are presented.

8.2 Rice

Rice, the world's most important cereal crop; it is the primary source of food and calories for about one-half the world population (Khush 2005). Rice plant growth and productivity is constantly exposed to a wide range of environmental stresses such as drought, salinity, extreme temperatures (heat and freezing) and submergence-reduced productivity, leading to significant crop losses globally (Bray et al. 2000). Exhibition of a distinct or a combination of intrinsic changes ascertains the capacity of a plant to sustain itself under unfavorable environmental conditions (Farooq et al. 2009); modified conditions in many metabolic, physiological and molecular pathways in plant growth can lead to serious yield losses (Bray et al. 2000; Kaplan et al. 2004; Sabehat et al. 1998; Sakamoto et al. 2004). Tolerance or susceptibility to these abiotic stresses is a very complex phenomenon, both because stress may occur

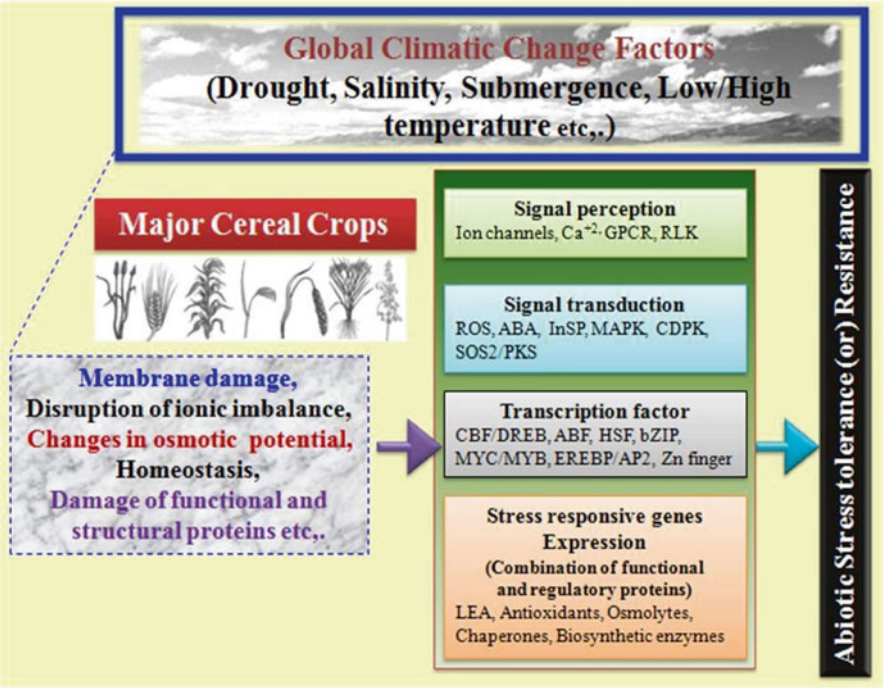

Fig. 8.1 A schematic representation of abiotic stress mechanisms and involvement of signal perception and transduction pathways in cereal crops (Source: Modified from Xiong et al. 2002)

at multiple stages of plant development and more than one type of stress simultaneously affects the plant. Therefore, the perception of abiotic stresses and signal transduction to switch on adaptive responses are critical steps in determining the survival and reproduction of plants exposed to adverse environments (Chinnusamy et al. 2004) (Fig. 8.1).

The affect of stresses on the rice plant can be divided into biotic and abiotic stresses, and they can be defined as any external factor exerting a negative impact upon the plant development (Taiz and Zeiger 1998). The rice plant responds to these environmental challenges through a number of defense mechanisms to maintain the optimal conditions; these involve changes at the whole plant, tissue, cellular, physiological and molecular levels for growth and development.

The availability of a rice genome sequence has lead to the identification of thousands of molecular markers, making map-based cloning a viable option for studies on functional genomics of rice (Jander et al. 2002). The genetic dissection of quantitative traits controlling the adaptive responses to abiotic stresses is a prerequisite to allow cost-effective applications of genomics-based approaches to breeding programs (Collins et al. 2008). In this context, QTL mapping is a powerful approach for locating genomic regions controlling complex traits (Gyenis et al. 2007). By linking phenotypic and genotypic data, QTL mapping enables the identification of the action, interaction, numbers and chromosomal locations of loci affecting particular traits (Miles and Wayne 2008) (Fig. 8.2).

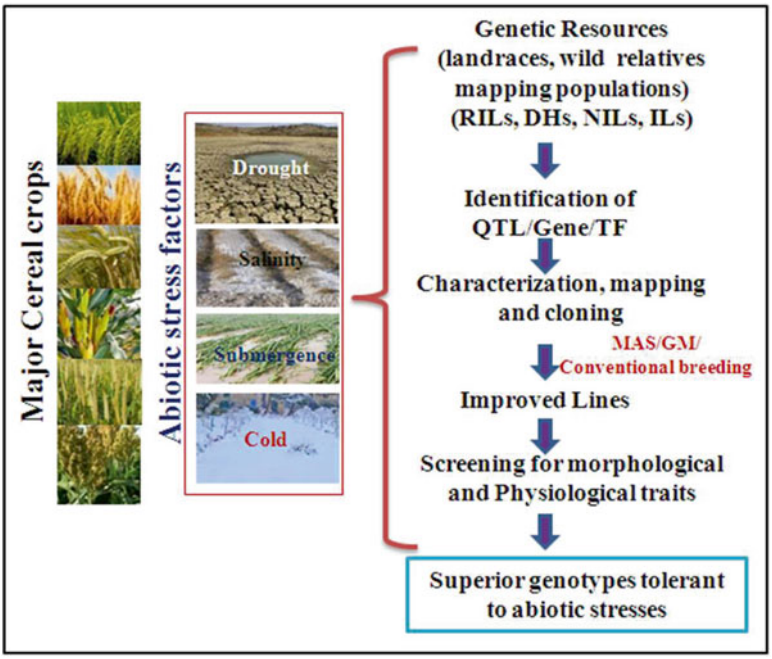

Fig. 8.2 A schematic diagram illustrating the approach for development of abiotic stress tolerance crops

The QTL mapping approach has been employed to dissect QTLs controlling the highly complex traits in the abiotic stress environment (Ren et al. 2005; Salvi and Tuberosa 2005; Zheng et al. 2003). QTLs have been detected in rice for many traits related to abiotic stress tolerance, including (**a**) drought (Chen et al. 2008a; Courtois et al. 2003; Lafitte et al. 2002; Liu et al. 2009; Price et al. 2002; Shunwu et al. 2012; Swamy et al. 2011); (**b**) low temperature (Baum et al. 2003; Francia et al. 2004; Jena et al. 2010; Lou et al. 2007; Reinheimer et al. 2004; Zeng et al. 2009); (**c**) salinity (Ammar et al. 2004; Islam et al. 2011; Sabouri and Sabouri 2008; Thomson et al. 2010); (**d**) submergence (Angaji et al. 2009; Neeraja et al. 2007; Septiningsih et al. 2012) and (**e**) heat tolerance (Cao et al. 2003; Chen et al. 2008b; Ye et al. 2012; Zhang et al. 2008). The QTLs associated with abiotic stress tolerance in cereals are given in Table 8.1.

8.2.1 Drought

Drought, salinity and other abiotic stresses are major causes of crop loss and this pattern is expected to increase worldwide due to global warming, leading to a loss of agriculturally-available land and reduced yields (Cominelli and Tonelli 2010). An increasing amount of research is being focused on developing crops that are resistant to abiotic stresses, such as drought, salinity, heat, cold, flooding, and

Table 8.1 QTLs associated with abiotic stress tolerance in cereals

Abiotic stress	Mapping population	Type	Trait	Crop	Chr	QTL	LOD	References
Submergence	IR74/Jalmagna	RILs	Initial plant height	Rice	1	QPh1a	10.1	Sripongpangkul et al. (2000)
Submergence	IR74/Jalmagna	RILs	Internode increment	Rice	1	QIne1	7.0	Sripongpangkul et al. (2000)
Drought	CT9993-5-10-1-M/ IR62266-42-6-2	DH	Cell membrane stability	Rice	8	qtl_8.2	5.6	Tripathy et al. (2000)
Submergence	IR74/Jalmagna	RILs	Initial plant height	Rice	1	QPh1a	10.1	Sripongpangkul et al. (2000)
Submergence	IR74/Jalmagna	RILs	Internode increment	Rice	1	QIne1	7.0	Sripongpangkul et al. (2000)
Submergence	FR13A/CT624	DH	21 days of seedling	Rice	9	Qtl.9	5.4	Kamolsukyunyong et al. (2001)
Cold	Nipponbare/Kasalath	RILs	Germination at 15 °C	Rice	4	qLTG	7.5	Miura et al. (2001)
Drought	CT9993/IR62266	DH	Osmotic adjustment	Rice	3	Osqtl	3.1	Zhang et al. (2001)
Drought	Bala/Azucena	F_6	Root-shoot ratio	Rice	1	Phqtl	4.6	Price et al. (2002)
Drought	CT9993/IR62266	DH	Deep root mass	Rice	1	Drqtl	3.52	Kamoshita et al. (2002)
Submergence	IR49830/CT6241	DH	Highly survival	Rice	1	qtl.1	5.4	Toojinda et al. (2003)
Submergence	FR13A/CT6241	RILs	Highly survival	Rice	9	qtl.9	65.8	Toojinda et al. (2003)
Drought	IAC165/Co39	RILs	Root thickness	Rice	1	Rtqtl	3.2	Courtois et al. (2003)
Submergence	IR49830/CT6241	DH	High tolerance	Rice	5	qtl.5	12.3	Toojinda et al. (2003)
Submergence	IR49830/CT6241	F_2-F_3	Fast elongation	Rice	9	qtl.9	16.2	Toojinda et al. (2003)
Drought	Zhenshan97B/IR AT109	RILs	Dry-weight of grains	Rice	2	Dwqtl	2.5	Liu et al. (2006)
Heat	996/4628	F_2-F_3	Flowering stage	Rice	4	qHsf	5.6	Govindaraj et al. (2005)
Drought	Zhenshan97B/IR AT109	RILs	Relative yield per plant	Rice	8	QRy8	3.4	Yue et al. (2006)
Drought	Zhenshan97B/IR AT109	RILs	Relative spikelet fertility	Rice	9	QRsf9	5.2	Yue et al. (2006)

Drought	Zhenshan97B/IR AT109	RILs	Relative rate of fertile panicles	Rice	12	QRfp12	4.2	Yue et al. 2006
Drought	Zhenshan97B/IR AT109	RILs	Relative grain weight	Rice	5	QRgw5	3.2	Yue et al. (2006)
Drought	Zhenshan97B/IR AT109	RILs	Relative rate of fertile panicles	Rice	12	QRfp12	4.2	Yue et al. 2006
Drought	Zhenshan97B/IR AT109	RILs	Relative grain weight	Rice	5	QRgw5	3.2	Yue et al. (2006)
Drought	Zhenshan97B/IR AT109	RILs	Relative yield per plant	Rice	8	QRy8	3.4	Yue et al. (2006)
Drought	Zhenshan97B/IR AT109	RILs	Relative spikelet fertility	Rice	9	QRsf9	5.2	Yue et al. (2006)
Submergence	IR49830-7/Swarna	BC_1F_1	14 days submergence	Rice	9	qtl.9	6.75	Neeraja et al. (2007)
Cold	AAV002863/ Zhenshan97B	DH	Seedling stage	Rice	1	qCtb1-a	6.33	Lou et al. (2007)
Cold	AAV002863/ Zhenshan97B	DH	Seedling stage	Rice	8	qCTS	5.91	Lou et al. (2007)
Heat	Zhongyouzao8/ZYZ8	RILs	Flowering stage	Rice	3	qHt3	7.1	Chen et al. (2008b)
Submergence	Khaiyan/IR64	BC_2F_1	Anaerobic germination	Rice	12	qAG-12	5.71	Angaji et al. (2009)
Salinity	Tarommahalli/Khazar	F_2	Na^+ uptake	Rice	1	qNAUP-1a	21.65	Sabouri and Sabouri (2008)
Heat	996/4628	F_2–F_3	Flowering stage	Rice	2	qhts-2	2.7	Chen et al. (2008)
Submergence	Khaiyan/IR64	BC_2F_1	Anaerobic germination	Rice	1	qAG-1	4.2	Angaji et al. (2009)
Salinity	Tarommahalli/Khazar	F_2	Seedling stage-chlorophyll content	Rice	3	qCHLC-3	20.35	Sabouri and Sabouri (2008)
Heat	996/4628	RILs	Flowering stage	Rice	5	qhts-5	4.6	Zhang et al. (2008)
Salinity	Tarommahalli/Khazar	F_2	K^+ uptake	Rice	3	qKUP-3	19.53	Sabouri and Sabouri (2008)
Salinity	Tarommahalli/Khazar	F_2	Fresh weight shoot	Rice	1	qFWSH-1	14.45	Sabouri and Sabouri (2008)
Cold	Asominor/IR24	RILs	Survival rate after 7 days at 6 °C	Rice	1	qCST	5.6	Jiang et al. (2008)
Submergence	Khaiyan/IR64	BC_2F_1	Anaerobic germination	Rice	11	qAG-11	3.92	Angaji et al. (2009)
Cold	Towada/MXBG	BC_3F_5	Young panicles to the milky mature stage	Rice	6	qCTB4-3	4.60	Zeng et al. (2009)

(continued)

Table 8.1 (continued)

Abiotic stress	Mapping population	Type	Trait	Crop	Chr	QTL	LOD	References
Salinity	CSR27/MI48	F_2-F_3	Reproductive stage in leaf	Rice	2	qClLR	7.57	Ammar et al. (2004)
Cold	Towada/KMXBG	BC_3F_5	Young panicles to the milky mature stage	Rice	3	qCTB4-3	5.23	Zeng et al. (2009)
Salinity	CSR27/MI48	F_2-F_3	Seedling stage in leafs	Rice	8	qNaLV	4.78	Ammar et al. (2004)
Heat	Bala/Azucena	RILs	Relative spikelet fertility	Rice	4	qtl_4	4.3	Jagadish et al. (2010)
Salinity	IR29/Pokkali	RILs	SES tolerance score	Rice	4	qSES4	3.4	Thomson et al. (2010)
Cold	Norin-PL8/Kirara397	F_2-F_3	Anther length	Rice	4	qCtb-1	8.2	Saito et al. (2010)
Salinity	IR29/Pokkali	BC_3F_5	Shoot Na^+ concentration	Rice	1	qSNC1	3.6	Thomson et al. (2010)
Salinity	IR29/Pokkali	BC_3F_5	Shoot Na-K ratio	Rice	1	qSNK1	7.6	Thomson et al. (2010)
Cold	IR66160-121-4-2-2/ Geumobyeo	RILs	Spikelet fertility	Rice	9	qPSST	5.4	Jena et al. (2010)
Heat	Bala/Azucena	RILs	Spikelet fertility	Rice	1	qtl_1.	6.2	Jagadish et al. (2010)
Cold	IR66160-121-4-2-2/ Geumobyeo	RILs	Days to heading	Rice	7	qPSST-	3.6	Jena et al. (2010)
Salinity	IR29/Pokkali	BC_3F_5	Leaf chlorophyll content	Rice	2	qCHL2	11.2	Thomson et al. (2010)
Cold	IR66160-121-4-2-2/ Geumobyeo	RILs	Culm length	Rice	3	qPSST	4.2	Jena et al. (2010)
Salinity	IR29/Pokkali	RILs	Initial SES tolerance score	Rice	3	qSES3	3.8	Thomson et al. (2010)
Heat	Bala/Azucena	RILs	Leaf rolling	Rice	1	qtl_1.	4.8	Jagadish et al. (2010)
Salinity	IR29/Pokkali	RILs	Seedling survival	Rice	1	qSUR1	3.2	Thomson et al. (2010)
Heat	Bala/Azucena	RILs	Grain yield	Rice	8	qtl_1.	5.6	Jagadish et al. (2010)
Salinity	IR29/Pokkali	RILs	Seedling height	Rice	2	qPH2	11.1	Thomson et al. (2010)
Drought	N22/Swarna	F_2	Harvest index	Rice	1	qDTHI1.1	18.5	Swamy et al. (2011)
Salinity	BRRI dhan40/ IR61920-3B-22-2-1	F_2-F_3	Seedling staged rice	Rice	8	SalTol8	7.01	Islam et al. (2011)

Salinity	BRRI dhan40/ IR61920-3B-22-2-1	F_2-F_3	Seedling staged rice	Rice	1	SalTol1	2.75	Islam et al. (2011)
Drought	N22/Swarna	F_2	Grain yield	Rice	1	qDTY1.1	13.4	Swamyet al. (2011)
Salinity	BRRI dhan40/ IR61920-3B-22-2-1	F_2-F_3	Seedling staged rice	Rice	10	SalTol10	4.52	Islam et al. (2011)
Drought	N22/IR64	F_2	Date of flowering	Rice	1	qDTF1.1	7.6	Swamy et al. (2011)
Drought	N22/MTU1010	F_2	Biomass yield	Rice	10	qDTF10.1	11.8	Swamy et al. (2011)
Heat	IR64/N22	BC_2F_1	Flowering stage	Rice	1	qHD3.1	5.4	Ye et al. (2012)
Submergence	R72/Madabaru	F_2	14 days submergence	Rice	2	qSUB2.1	3.8	Septiningsih et al. (2012)
Heat	IR64/N22	F_2-F_3	Panicle length	Rice	9	qPL9.1	4.02	Ye et al. (2012)
Heat	IR64/N22	BC_2F_1	Flag leaf length	Rice	1	qFLL1.1	8.55	Ye et al. (2012)
Submergence	R72/Madabaru	F_2	14 days submergence	Rice	1	qSUB1.1	9.4	Septiningsih et al. (2012)
Submergence	R72/Madabaru	F_2	14 days submergence	Rice	9	qSUB9.1	3.6	Septiningsih et al. (2012)
Heat	IR64/N22	BC_2F_1	spikelet fertility	Rice	3	qHD1.1	6.76	Ye et al. (2012)
Submergence	R72/Madabaru	F_2	14 days submergence	Rice	12	qSUB12.1	4.2	Septiningsih et al. (2012)
Heat	N22/IR64	BC_2F_2	Plant height	Rice	1	qPH1.1	42.43	Ye et al. (2012)
Heat	N22/IR64	BC_2F_2	Spikelet number	Rice	1	qPH1.1	6.01	Ye et al. (2012)
Heat	N22/IR64	F_2	Spikelet fertility	Rice	4	qHTSF4.1	6.66	Ye et al. (2012)
Heat	N22/IR64	BC_2F_2	Heading date	Rice	3	qHD3.1	4.24	Ye et al. (2012)
Submergence	Forno/Oberkulmer	RILs	Germination under flooding	Wheat	4B	qtl.4	3.6	Burgos et al. (2001)
Submergence	Forno/Oberkulmer	RILs	Germination under flooding	Wheat	6A	qtl.6	3.0	Burgos et al. (2001)
Submergence	Forno/Oberkulmer	RILs	Germination under flooding	Wheat	2A	qtl.2	4.2	Burgos et al. (2001)
Drought	Dharwar Dry/Sitta	RILs	Grain yield	Wheat	3	grqtl.3	5.74	Kirigwi et al. (2007)
Drought	Dharwar Dry/Sitta	RILs	Biomass	Wheat	3	grqtl.3	9.70	Kirigwi et al. (2007)

(continued)

Table 8.1 (continued)

Abiotic stress	Mapping population	Type	Trait	Crop	Chr	QTL	LOD	References
Drought	Hanxuan 103/Lumai 14	DH	Flowering stage	Wheat	4A	qSwscf.1	10.26	Yang et al. (2009)
Drought	Hanxuan 103/Lumai 14	DH	Grain-filling	Wheat	6A	qSwscg	5.29	Yang et al. (2009)
Drought	Hanxuan 103/Lumai 14	DH	Thousand-grain weight	Wheat	6B	qReswc	4.18	Yang et al. (2009)
Salinity	Berkut/Krichauff	DH	Sodium exclusion	Wheat	6A	qtl.6	3.8	Genc et al. (2010)
Drought	C306/HUW206	RILs	Physiological traits	Wheat	3A	qtl.3	3.5	Kumar et al. (2012)
Drought	C306/HUW206	RILs	Physiological traits	Wheat	4B	qtl.4	3.2	Kumar et al. (2012)
Cold	M808/CS	RILs	Germination stage	Wheat	5A	WCBF2	5.86	Motomura et al. (2013)
Cold	M808/CS	RILs	7 days of old seedling	Wheat	5A	Wlt10	3.03	Motomura et al. (2013)
Cold	M808 / CS	RILs	Germination stage	Wheat	4B	Wcor14		Motomura et al. (2013)
Cold	Nure/Tremois	DH	Winter survival (field)	Barley	5H	qtl.5	6.7	Francia et al. (2004)
Cold	Nure/Tremois	DH	Frost tolerance	Barley	5H	qtl.5	9.2	Francia et al. (2004)
Cold	Nure/Tremois	DH	COR14b accumulation	Barley	6H	qtl.6	3.2	Francia et al. (2004)
Drought	Yerong/Franklin	DH	Grains per spike	Barley	2H	GSw2.1	5.63	Xue et al. (2010)
Drought	Yerong/Franklin	DH	Plant height	Barley	6H	PHw2.1	5.52	Xue et al. (2010)
Drought	Yerong/Franklin	DH	Grain yield	Barley	7H	GYw1.2	7.45	Xue et al. (2010)
Drought	Yerong/Franklin	DH	Spikes per plant	Barley	7H	SPw1.1	3.31	Xue et al. (2010)
Salinity	Steptoe/Morex	DH	Ion homeostasis	Barley	2H	qtl.2	5.2	Nguyen et al. (2013)
Salinity	Steptoe/Morex	DH	Shoot K^+	Barley	7H	qtl.7	6.81	Nguyen et al. (2013)
Salinity	Steptoe/Morex	DH	Root dry weight	Barley	3H	qtl.3	5.71	Nguyen et al. (2013)
Submergence	B64/teosinte	F_2-F_3	Root aerenchyma formation	Maize	1	RAqtl	3.0	Mano et al. (2007)
Submergence	B64/teosinte	F_2-F_3	Root aerenchyma formation	Maize	5	RAqtl	3.6	Mano et al. (2007)
Drought and heat	Zong3/87-1	RILs	Shoot fresh weight	Maize	1	qRSFW1	3.5	Liu et al. (2011)

Drought and heat	Zong3/87	RILs	Shoot dry weight	Maize	2	qRSDW2	3.1	Liu et al. (2011)
Drought and heat	Zong3/87	RILs	Leaf tolerance	Maize	9	qLTD9-1	4.6	Liu et al. (2011)
Drought and heat	Zong3/87	RILs	Leaf tolerance	Maize		qLTD10	3.6	Liu et al. (2011)
Drought	CML444/MALAW1	RILs	Grain yield	Maize	1	qtl1.1	7.35	Gustavo et al. (2013)
Drought	CML444/MALAW1	RILs	Anthesis-silking	Maize	2	qtl1.2	5.34	Gustavo et al. (2013)
Drought	CML440/CML504	F_2-F_3	Anthesis-silking	Maize	6	qtl1.6	4.37	Gustavo et al. (2013)
Drought	CML440/CML504	F_2-F_3	Grain yield	Maize	3	qtl1.3	11.19	Gustavo et al. (2013)
Drought	CML440/CML504	F_2-F_3	Grain yield	Maize	3	qtl1.3	3.18	Gustavo et al. (2013)
Drought	HZ32/K12	F_2-F_3	Physiological traits	Maize	4	ph4-1	3.91	Khalid et al. (2013)
Drought	HZ32/K12	F_2-F_3	Physiological traits	Maize	6	ph6-1	3.97	Khalid et al. (2013)
Drought	HZ32/K12	F_2-F_3	Physiological traits	Maize	6	tdw6-1	4.21	Khalid et al. (2013)
Drought	HZ32/K12	F_2-F_3	Physiological traits	Maize	7	tdw7-2	5.91	Khalid et al. (2013)
Cold	Shan Qui Red/SRN39	RILs	germination and seedling stage	Sorghum	2	qtl.2	4.2	Knoll et al. (2008)
Drought	H77/833-2/PRLT2/89-33	RILs	Transpiration, water use leaf traits	Pearl millet	1	qtl.1	3.7	Kholova et al. (2012)
Drought	H77/833-2/PRLT2/89-33	RILs	Leaf dry weight	Pearl millet	2	qtl.2	4.5	Kholova et al. (2012)
Drought	H77/833-2/PRLT2/89-33	RILs	Shoot dry weight	Pearl millet	7	qtl.7	8.6	Kholova et al. (2012)

DH Double haploid, *RIL* Recombinant inbred line

nutrient limitation (Collins et al. 2008; Witcombe et al. 2008). Of these stresses, drought is the most serious constraint to rice production in less favorable rice-growing areas and most of the popular farmers' varieties are susceptible (Serraj et al. 2009). Depending on the timing and severity of drought stress at the crop developmental stage, drought can be categorized into three types: vegetative, intermittent and terminal (Chang et al. 1979; Fischer et al. 2003; Kamoshita et al. 2008). Of these three, the occurrence of terminal drought stress at the reproductive stage during the cropping season directly affects grain yield and has devastating consequences (Fukai et al. 2001; Lanceras et al. 2004; Tsubo et al. 2006; Venuprasad et al. 2009). The severity of drought stress levels depends on various factors such as soil type, evaporation, transpiration rate, temperature, relative humidity and rainfall pattern (Fischer et al. 2003). The major drawbacks for the development of drought-tolerance cultivars include lack of effective selection criteria for drought tolerance related traits such as morphological, physiological traits and molecular mechanisms of genes and low heritability of grain yield under stress; these are key reasons for the slow progress in drought tolerance in plant breeding (Ouk et al. 2006).

8.2.2 MAS Approaches in QTL and Genes

Marker assisted selection (MAS) is one of the best approaches for the development of drought tolerance (DT) in rice, a major target for the plant breeders and biotechnologists (Jongdee et al. 2002). For effective implementation of MAS in a rice-breeding program for drought tolerance, targeted QTL/gene must have a large effect on grain yield under stress and be consistently expressed in different backgrounds. Therefore, it is imperative to identify background-independent drought-tolerance QTLs from rice germplasm resources and corresponding tightly-linked molecular markers for developing DT cultivars by MAS (Fig. 8.2) (Table 8.2). However, the successful use of QTLs in marker-assisted selection depends on their effect and consistency across genetic backgrounds and environments. Most of the QTLs for grain yield under drought have been mapped against a single genetic background in early-segregating generations (F_3, BC_2 and BC_2F_2) and were evaluated in a limited number of environments (Swamy et al. 2011).

QTL mapping has been carried out in an attempt to determine the genetic basis of several traits related to drought tolerance, including the following: (**a**) deep rooting (Ekanayake et al. 1985; Lilley and Fukai 1994; Pantuwan et al. 2002; Wade et al. 2000); (**b**) osmotic adjustment (Babu et al. 2003; Jongdee and Cooper 1998; Ludlow and Muchow 1990; Robin et al. 2003; Zhang et al. 1999; Zhu et al. 1997); (**c**) cell membrane stability (Tripathy et al. 2000); (**d**) abscisic acid (ABA) content (Quarrie et al. 1994, 1997); (**e**) stomatal regulation Price and Tomos 1997; (**f**) leaf water status and root morphology (Ali and Awan 2004; Champoux et al. 1995; Courtois et al. 2000; Kamoshita et al. 2002; Price and Tomos 1997; Price et al. 2002; Ray et al. 1996; Yadav et al. 2002; Zhang et al. 2001; Zheng et al. 2000); (**g**) root thickness (Courtois et al. 2003); (**h**) root dry weight, pulling force, and root length and ratio of shoot and

Table 8.2 Abiotic stress tolerance genes/transcription factors in cereal and other crops

Abiotic stress tolerance levels	Genes/transcription factor	Crop	References
Drought and salinity	PSP 1015	Wheat	Hollung et al. (1994)
Cold	Tacr7	Wheat	Gana et al. (1997)
Cold tolerance	OscodA	Rice	Sakamoto and Alia Murata (1998)
Salinity	Mn-SOD	Rice	Tanaka et al. (1999)
Salinity	OsA1/ OsA2	Rice	Zhang et al. (1999)
Drought, cold and salinity	ABF4	*Arabidopsis thaliana*	Choi et al. (2000)
Salinity and cold	GS2	Rice	Hoshida et al. (2000)
Cold	ScRPS7	Wheat	Berberich et al. (2000)
Osmotic and drought	TaSAMDC	Wheat	Li and Chen (2000)
Cold	CHT9	Wheat	Yet et al. (2000)
Drought and salinity	OsCDPK7	Rice	Saijo et al. (2000)
Salinity	OsGS2	Rice	Hoshida et al. (2000)
Drought, Cold and salinity	OsCDPK7	Rice	Sajjo et al. (2000)
Cold tolerance	OsCtb1	Rice	Saito et al. (2001)
Water logging	Pdc1	Rice	Rahaman et al. (2001)
Salinity and drought	Osmotin	Rice	Barthakur et al. (2001)
Cold	AtCBF2	Tomato	Hsieh et al. (2002)
Drought	PDH	*A. thaliana*	Mani et al. (2002)
Cold tolerance	MYBS3	Rice	Jang et al. (2003)
Drought and cold	TaDREB1	Wheat	Shen et al. (2003a)
Drought and salinity	AhDREB1	Tobacco	Shen et al. (2003b)
Cold	OsDREB1A,	Rice	Dubouzet et al. (2003)
Salinity and dehydration	OsDREB2A	Rice	Dubouzet et al. (2003)
Drought	mtlD	Wheat	Abebe et al. (2003)
Drought	betA	Maize	Quan et al. (2004)
Drought and salinity	RD26	*A. thaliana*	Fujita et al. (2004)
Drought and freezing	NPK1	Maize	Shou et al. (2004)
Salinity	OsNHX1	Rice	Fukuda et al. (2004)
Drought	TaLTP1	Wheat	Pellegrineschi et al. (2004)
Drought	DREB1A	Wheat	Jang et al. (2004)
Drought and salinity	OsRacB	Rice	Luo et al. (2006)
Salinity	TVP1	Wheat	Brini et al. (2005)
Salinity	OsHKT1	Rice	Kader et al. (2006)
Drought, cold and salinity	OsDREB1B	Rice	Ito et al. (2006)
Drought, cold and salinity	WDREB2	Wheat	Egawa et al. (2006)
Salt and submergence	AtMYB44	*A. thaliana*	Yanhui et al. (2006)

(continued)

Table 8.2 (continued)

Abiotic stress tolerance levels	Genes/transcription factor	Crop	References
Cold and heat	OsDREB1B	Rice	Qin et al. (2007)
Salinity, drought and cold	OsDREB1F	Rice	Wang et al. (2008)
Drought	TPS1-TPS2	*Nicotiana tabacum*	Karim et al. (2007)
Drought and cold	HvCBF4	Rice	Oh et al. (2007)
Salinity	AeNHX1	Wheat	Qiao et al. (2007)
Drought	ZmNF-YB2	Maize	Nelson et al. (2007)
Drought	OsDREB1G	Rice	Chen et al. (2008)
Drought and cold	Wlip19	Wheat	Kobayashi et al. (2008)
Drought and salinity	OsAB15	Rice	Zou et al. (2008)
Drought and salinity	OsbZIP23	Rice	Xiang et al. (2008)
Drought	CodA	*Solanum tuberosum*	Ahmad et al. (2008)
Drought	Rab16D	Rice	Zou et al. (2008)
Cold tolerance	HOS10	Rice	Chen et al. (2008)
Drought	TsVP	Maize	Li et al. (2008)
Cold	GmWRKY21	*A. thaliana*	Zou et al. (2008)
Salinity	OsWRKY45-2	Rice	Tao et al. (2009)
Drought	Os AP59 /Os AP37	Rice	Oh et al. (2009)
Drought, heat and salinity	ZmbZIP17	Maize	Jia et al. (2009)
Salinity	CaZF	Tobacco	Jain et al. (2009)
Drought and salinity	ONAC045	Rice	Zheng et al. (2009)
Salinity and drought	OsDHODH1	Rice	Liu WY et al. (2009)
Submergence	SNORKEL1/ SNORKEL2	Rice	Hattori et al. (2009)
Drought	Dro1	Rice	Uga et al. (2011)
Drought, cold and salinity	OsABF2	Rice	Hossain et al. (2010)
Salinity and cold	TaSnRK	Wheat	Zhang et al. (2010)
Drought cold and salinity	OsDREB1	Rice	Fukao et al. (2011)
Hyperosmotic	PgDREB2A	Tobacco	Agarwal et al. (2010)
Drought	OsNAC10	Rice	Jeong et al. (2010)
Cold	Osmyb4	Rice	Park et al. (2010)
Drought and salinity	OsAREB1	Rice	Jin et al. (2010)
Drought	Oshrf1	Rice	Zhang et al. (2011)
Drought and cold	SbDREB2	Sorghum	Bihani et al. (2011)
Drought and salinity	OSRIP18	Rice	Jiang et al. (2012)
Drought and submergence	OsERF3	Rice	Wan et al. (2011)

(continued)

Table 8.2 (continued)

Abiotic stress tolerance levels	Genes/transcription factor	Crop	References
Drought	SbDREB2	Sorghum	Bihani et al. (2011)
Drought and salinity	OsRIP18	Rice	Jiang et al. (2012)
Drought	OsGRF8	Rice	Choi et al. (2004)
Osmotic tolerance	OsGRF8	Rice	Choi et al. (2004)
Drought and salinity	OsHsfA7	Rice	Liu et al. (2013)
Cold	Wlt10	Wheat	Motomura et al. (2013)
Cold	Wdhn13	Wheat	Motomura et al. (2013)
Cold	Wcor14	Wheat	Motomura et al. (2013)

root (Champoux et al. 1995; Nguyen et al. 2004; Price et al. 2002; Robin et al. 2003; Uga et al. 2011; Zhang et al. 2001; Zheng et al. 2000); (**i**) stomatal closure (Lv et al. 2007); (**j**) relative water content (Altinkut et al. 2001; Colom and Vazzana 2003); (**k**) photosynthetic rate (Hetherington and Woodward 2003; Woodward et al. 2002); (**l**) chlorophyll stability index (Ananthi and Vanangamudi 2013); (**m**) leaf rolling (Hsiao 1973); (**n**) proline accumulation (Gunes et al. 2005; Shao et al. 2005); (**o**) signal transduction, osmoregulation and antioxidant systems (Hare et al. 1999; Kishor et al. 2005; Szabados and Savoure 2009); (**p**) stomatal conductance and transpiration rate (Chaves and Oliveira 2004; Lawlor and Cornic 2002); (**q**) grain yield and yield components (Lafitte et al. 2004; Xu et al. 2005); (**r**) stay green (Jiang et al. 2004); (**s**) canopy temperature, leaf rolling and leaf drying (Yue et al. 2006); (**t**) grain yield (Kirigwi et al. 2007; Salem et al. 2007) and (**u**) thousand grain weight (Nezhad et al. 2012), are all associated to some degree with drought tolerance.

Since drought tolerance characters are quantitative in nature, the complete genetic dissection of these complex traits into component genetic factors is a preliminary task. Many drought-related QTLs have been identified in major cereal crops that can be effectively used in breeding programs. This necessitates that more and more replicated yield tests should be conducted in order to accurately characterize their effects and to evaluate their stability across different environments (Cattivelli et al. 2008) (Table 8.1).

8.2.2.1 Tolerance to High Temperature

Heat stress, especially during the flowering stage in rice, affects the seed setting rate and floret sterility, resulting in high yield losses (Morita et al. 2005; Peng et al. 2004). In a climate-change scenario, when day temperatures are predicted to rise to alarming levels, research on the genetic mechanisms of heat tolerance becomes more relevant and the development of new rice varieties with heat tolerance gene(s) are a high-priority area. The main cause of spikelet sterility induced by high temperature at the flowering stage is anther indehiscence. The anthers of heat-tolerant cultivars dehisce more easily than those of susceptible cultivars under high temperature conditions (Mackill et al. 1982; Matsui et al. 1997a, b, 2001; Satake and Hayase

1970). This is because of the tight closure of the locules by the cell layers, which delays locule opening and decreases spikelet fertility (Matsui and Omasa 2002); it has been suggested that spikelet fertility at high temperature can be used as a screening tool for heat tolerance during the flowering stage (Prasad et al. 2006).

In recent years, with the development of molecular-marker techniques and their widespread application, there were reports of genetic research on heat tolerance in rice by utilizing molecular markers (Cao et al. 2003; Zhao et al. 2006). Much effort has been made to carry out molecular mapping of heat tolerance quantitative trait loci (QTLs) in rice at the booting, flowering and grain filling to ripening stages (Jagadish et al. 2010; Xiao et al. 2011; Ye et al. 2012; Zhang et al. 2008) and QTL mapping studies for heat tolerance have been conducted on various rice populations at the booting (Zhao et al. 2006) and flowering stages (Cao et al. 2003; Chen et al. 2008; Jagadish et al. 2010; Xiao et al. 2011; Zhang et al. 2008). However, confirmation and fine mapping of the identified QTL for heat tolerance have not been reported. Using identified genetic resources and QTLs to improve heat tolerance in rice varieties has not been achieved. Further studies on QTL mapping using accurate phenotyping technology and validation of mapped major QTLs in different populations are needed to identify candidates for map-based cloning of genes which account for heat tolerance in rice (Table 8.1).

8.2.2.2 Cold Tolerance

Chilling injury is one of the most important limiting factors affecting rice production in temperate and high-elevation areas. Rice is sensitive to low temperature and its growth and production is reduced dramatically at low temperature (Lou et al. 2007). Good cold tolerance at the seedling stage is an important character for stable rice production, especially in direct-seeded fields. Development of varieties having tolerance to cold is one of the effective ways to avoid the low temperature damage.

Low temperature often affects plant growth and productivity, which causes significant crop yield losses (Xin and Browse 2001). Plants differ in their tolerance to chilling (0–15 °C) and freezing (<0 °C) temperatures. In general, plants from temperate climatic regions are considered to be chilling tolerant to a variable degree, and can increase their freezing tolerance by being exposed to chilling, non-freezing temperatures, a process known as cold acclimation, which is associated with biochemical and physiological changes (Gilmour et al. 2000; Shinozaki and Yamaguchi-Shinozaki 1996; Thomashow 1998) and ultimately exhibited marked changes in gene expression, bio membrane lipid composition and small molecule accumulation (Yamaguchi-Shinozaki and Shinozaki 2006). Conventional breeding methods have met with limited success in improving the cold tolerant crops due to the complexity of stress tolerance traits, low genetic variance of yield components under stress condition and lack of efficient selection criteria. It is important, therefore, to look for alternative strategies to develop cold stress tolerant crops.

Biotechnology offers new strategies that can be used to develop transgenic crop plants with improved tolerance to cold stress. Molecular markers have facilitated

the identification of chromosomal regions associated with many complex traits in rice including tolerance to low temperature. Identification of QTLs related to low temperature tolerance in rice have been reported by utilizing RFLP markers (Harushima et al. 1998; Takeuchi et al. 2001) and microsatellite markers (Andaya and Mackill 2003; Fujino et al. 2004; Kuroki et al. 2007; Lou et al. 2007; McCouch et al. 2002; Suh et al. 2010).

Saito et al. (1995) identified two QTLs responsible for the cold tolerance at the booting stage which were mapped on chromosomes 3 and 4 in populations derived from Norin/Pl8 cross combination. Fine mapping of the QTL on chromosome 4 has identified two genes (Ctb1 and Ctb2) for cold tolerance in a 56 kb region (Saito et al. 2001). The QTL for cold tolerance on chromosome 4 of Norin/PL8 cross combination consists of two closely linked genes, related to cold tolerance and anther length, and Ctb1 was delimited to seven open reading frames (ORFs) in a 56-kb region (Saito et al. 2004). Several QTLs linked to cold tolerance at the reproductive stage were mapped on different chromosomes using F_2, BC_5F_3 and doubled-haploid (DH) populations (Andaya and Mackill 2003; Dai et al. 2004; Liu et al. 2003; Saito et al. 2001; Suh et al. 2010; Xu and Crouch 2008; Ye et al. 2010). A QTL for cold tolerance between RM5647 and PLA61 on chromosome 8 explains 26.6 % of the phenotypic variance, and its additive effect is 11.4 % (Kuroki et al. 2007). The QTLs reported on chromosomes 1, 2, 3, 5, 6, 7, 9 and 12 explain 11–17 % of the phenotypic variance identified to confer cold tolerance at the booting stage (Andaya and Mackill 2003). Consequently, it is crucial to screen for cold tolerance at this stage and to understand the genetic basis and molecular mechanisms of cold tolerance. Despite many QTL mapping reports on cold tolerance at the booting stage, cold tolerance-related traits have not been studied in large populations over time and at different locations (Table 8.1).

8.2.2.3 Submergence

Submergence stress is a widespread problem in rice-growing areas, especially in the flood-prone rainfed lowlands and it was reported that 22 million ha per year of in South and Southeast Asia have been affected at various stages (Jackson and Ram 2003; Ram et al. 2002). Different flooding types include flooding during seed germination (anaerobic germination), flash flooding (submergence), stagnant flooding and deeper stagnant flooding. In the field, more than one of these situations can occur in the same season or in different seasons (Ismail et al. 2009). Plants subjected to submergence stress are exposed to low light, limited gas diffusion, effusion of soil nutrients, mechanical damage and increased susceptibility to pests and diseases that ultimately affect the grain yield (Ram et al. 2002).

Submergence itself can be classified into flash flooding and deepwater flooding according to the duration of flooding and the water depth (Bailey-Serres et al. 2010; Jackson and Ram 2003). Flash flooding, which generally lasts only a few weeks, is caused by heavy rain but standing water is only present for a short duration. On the other hand, deepwater flooding, extending for several months, occurs during the

rainy season, and with standing water levels reaching several meters (Hattori et al. 2011). Flooding can cover the entire plant for a prolonged period; most rice cultivars die within 7 days of complete submergence (Bailey-Serres et al. 2010; Xu et al. 2006). A limited number of rice cultivars overcome submergence through antithetical growth responses.

Recent studies provide insights into the hormonal control of submergence tolerance. According to Fukao and Bailey-Serres (2008), under submerged conditions, the concentration of ethylene increases while the concentration of abscisic acid (ABA) decreases. In intolerant plants, this triggers an increase in gibberellic acid (GA) and the induction of cell elongation. In tolerant plants, the ethylene-induced increase in SUB1A expression and inhibits the accumulation of GA by increasing the accumulation of the GA-signaling suppressors thus decreasing the GA responsiveness. Under complete submergence, the presence of SUB1A in the submergence-tolerant genotypes suppresses the perception and production of ethylene via its induction of the Slender Rice-1 (SLR1) and SLR Like-1 (SLRL1) genes (Fukao and Bailey-Serres 2008).

Deepwater rices responds to submergence by promoting internode elongation to outgrow floodwater levels. This escape response is regulated by a polygenic locus that encodes two APETALA2/ Ethylene Response Factor (AP2/ERF) DNA binding proteins, SNORKEL 1 (SK1) and SNORKEL 2 (SK2) (Hattori et al. 2011). The AP2/ERF super family of rice can be divided into three families based on sequence similarity and number of domains: AP2, ERF and RAV. Of these, the ERF family is classified into 14 groups (I–XIV) based on gene structures, phylogeny and conserved motifs (Nakano et al. 2006).

Submergence tolerance is controlled by a single major QTL on chromosome 9, along with a number of minor QTLs (Toojinda et al. 2003; Xu and Mackill 1996). All studies on submergence have used the traditional genotype FR13A, which is one of the most submergence-tolerant donor varieties. The major QTL, named Sub1, with a LOD score of 36 and an R2 value of 69 % that provides tolerance to complete submergence for up to 2 weeks, has been identified as a major determinant of submergence tolerance in rice variety FR13A and its derived progenies (Xu and Mackill 1996). More recently, Sub1 has been fine-mapped and cloned, yielding three candidate genes, Sub1A, Sub1B and Sub1C (Fukao et al. 2006; Ruanjaichon et al. 2008; Xu et al. 2006).

Sub1A containing an ethylene-response-factor (ERF) domain, was first cloned from rice variety FR13A (Flood Resistant 13A) (Xu et al. 2006). The ethylene-response-factor (ERF) domain composed of a super family of transcription factors in plants and microorganisms (Nakano et al. 2006; Okamura et al. 1997). EREBPs are characterized by the presence of the highly-conserved EREBP DNA-binding domain of about 60 amino acids (Okamura et al. 1997). Plant proteins that contain ERF domains are known regulators of abiotic and biotic stress responses (Gutterson and Reuber 2004; McGrath et al. 2005; Rahman et al. 2001). Within the sequenced Sub1 locus of an FR13A derived tolerant breeding line, three putative ERF genes were identified and designated SUB1A-1, SUB1B-1, and SUB1C-1 (Xu et al.

2006). ERF family members control diverse biological functions in growth and development such as leaf epidermal cell density, flower development and embryo development (Boutilier et al. 2002; Elliott et al. 1996), as well as hormonal signaling mediated by ethylene, cytokinin (Rashotte et al. 2006) and brassinosteroid (Alonso et al. 2003; Hu et al. 2004). ERFs are also involved in response to biotic and abiotic stimuli such as pathogen infection, drought and freezing stresses (Gilmour et al. 2000; Hao et al. 1998; Liu et al. 1999).

The SUB1A gene is absent from the Nippon bare reference genome and all other analyzed *Oryza sativa japonica* varieties due to an inversion and deletion. In intolerant *O. sativa indica* varieties, the SUB1A gene is either absent or present as the allelic variant SUB1A-2. Single nucleotide polymorphisms (SNPs) were also identified for the SUB1C gene, distinguishing the tolerant SUB1C-1 allele from those found in intolerant varieties (SUB1C-2–SUB1C-7). In contrast, no tolerant-specific allele for SUB1B was identified (Xu et al. 2006). Gene expression analyses showed that all three ERF genes were induced under submergence and revealed a specifically high SUB1A and low SUB1C expression in tolerant varieties. SUB1A had been subsequently identified as the major determinant of tolerance by a transgenic approach showing that constitutive expression of the SUB1A-1 allele conferred tolerance of submergence to an intolerant variety (Liaogeng) that naturally lacks the SUB1A gene (Xu et al. 2006).

A breakthrough in the development of submergence-tolerant varieties was facilitated by the fine-mapping and sequencing of the Sub1 locus (Collard and Mackill 2008; Septiningsih et al. 2009; Toojinda et al. 2003; Xu and Mackill 1996). Accordingly, precise gene-based markers have been designated as SUB1 and used for its successful introgression into popular rice varieties (Neeraja et al. 2007; Septiningsih et al. 2009; Siangliw et al. 2003; Singh et al. 2009; Toojinda et al. 2003). This SUB1gene was transferred into six Asian rice mega-varieties (those widely grown in Asia), namely: Swarna, Samba Mahsuri, IR64, BR11, Thadokkam 1 (TDK1) and CR1009. These mega-varieties, possessing agronomic and quality traits preferred by farmers, were introgressed with the SUB1 gene using marker-assisted backcrossing (MABC) (Mackill et al. 2006; Neeraja et al. 2007; Septiningsih et al. 2009). When the SUB1A locus of FR13A was introgressed into the rice variety Swarna via marker-assisted selection, the resultant new variety showed enhanced submergence tolerance.

8.3 Wheat

8.3.1 Drought

Because wheat (*Triticum aestivum* L.) is the cereal of choice in most countries, constant efforts are needed to boost its production to keep pace with the ever-increasing population. But unfortunately, these efforts are being hampered by a

number of abiotic stresses among which drought is most important (Boyer 1982). According to Pfeiffer et al. (2005), 50 % of wheat production area is affected by drought worldwide. Drought leads to abnormal germination and poor crop stand (Harris et al. 2002; Kaya et al. 2006). Furthermore, drought stress at various critical growth stages like flowering and grain filling greatly reduces crop yield and for that reason its importance has been recognized at the global level. Thus, developing drought-resistant cultivars has been a major objectives of plant breeders and plant biotechnologists.

Although considerable efforts have been made to develop drought-tolerant cultivars of wheat through conventional breeding approaches, they met with little success due to the quantitative (polygenic) nature of drought tolerance, which is more influenced by external environmental conditions than by the genetic component (Ingram and Bartels 1996; Zhang 2004). The affects of drought stress on plant growth and development have been observed to impact cells, tissue and organs (Beck et al. 2007) and their mechanisms influence major regulations at the morphological, physiological and molecular levels (Farooq et al. 2009; Xiong et al. 2002).

Due to the increased understanding of gene structure and function at the cellular and molecular levels (Gosal et al. 2009), genomic-based approaches can now provide excellent opportunities to search and map QTLs for drought tolerance. Previous reports indicated that various DNA markers such as RFLP, AFLP and SSR have been used to tag QTLs for drought stress in wheat (Quarrie et al. 2005). Kirigwi et al. (2007) used SSR and EST markers for mapping QTL on chromosome 4A for grain yield and yield components in wheat. The markers associated with the QTL were XBE637912, Xwmc89, and Xwmc420. Quarrie et al. (2005) conducted mapping of QTLs for drought tolerance in hexaploid wheat which were located on chromosomes 1A, 1B, 2A, 2B, 2D, 3D, 5A, 5B, 7A and 7B. Thus, the DNA markers closely linked with QTLs conferring drought tolerance would greatly enhance the selection efficiency (Cattivelli et al. 2008).

8.3.2 MAS Approaches

8.3.2.1 Cold Stress

Cold/freezing temperatures represent one of the significant abiotic stresses limiting geographical distribution of plants and reducing crop quality and productivity. Freezing tolerance, one of these complex traits, is acquired through the cold acclimation process in many overwintering plants of temperate regions (Thomashow 1999).

A large number of genes with various functions are induced during cold acclimation (Rabbani et al. 2003; Seki et al. 2002). In particular cold-responsive (Cor)/late-embryogenesis-abundant (Lea) genes are transcriptionally activated in cold acclimation, and the accumulated COR/LEA proteins lead to protection of the integrity of cell structures and functions from freezing damage (Kosova et al. 2010; Thomashow 1999). Most Cor/Lea genes, including Wlt10, Wdhn13 and Wcor14, show differential expression levels in two wheat cultivars with contrasting levels of

freezing tolerance under low temperature conditions (Kobayashi et al. 2004; Ohno et al. 2003; Tsvetanov et al. 2000). Over-expression of the wheat CBF/DERB transcription factors increases freezing tolerance of transgenic plants (Kobayashi et al. 2008; Morran et al. 2011). The CBF regulon controls one of the important regulatory pathways in development of freezing tolerance in wheat (Winfield et al. 2010).

In common wheat, Vrn-1 and Fr-1 are well-known major quantitative trait loci determining the vernalization requirement for flowering and winter hardiness. The two loci are linked on the long arms of homoeologous group 5 chromosomes (Galiba et al. 1995; Sutka et al. 1999). The CRT/DRE, ABRE and other cold-responsive motifs have been identified in the 5′ upstream regions of many Cor/Lea genes in common wheat. Wcbf2 genes encode a putative transcription factor recognizing the CRT motif (Takumi et al. 2003).

The WCBF2 cascade is tightly associated with cold acclimation and the Fr-1 alleles, whereas expression of the cold-responsive Vrn-1 candidate gene WAP1 is apparently not related to expression of the Wcbf2 gene (Kobayashi et al. 2005). Expression of other cold-responsive transcription factor genes, Wdreb2 and Wlip19, are weakly controlled by Fr-1, while Wabi5 transcription factor gene involved in the ABA-dependent pathway appears to act independently of Fr-1 (Kobayashi et al. 2004). Mutant analyses suggest that ABA sensitivity is not associated with cold acclimation, while ABA is involved in determination of the basal level of freezing tolerance in wheat. Thus, the CBF-mediated Cor/Lea gene expressions play central roles in cold acclimation to develop high levels of freezing tolerance in wheat.

8.3.2.2 Salinity

Salt tolerance in cereals is known to be associated with the control of shoot Na^+ content; with tolerant lines having more efficient systems to exclude sodium from their shoots. A single locus (Kna1) localized on chromosome 4D of wheat was shown to control K^+/Na^+ discrimination and in a saline environment, bread wheat (AABBDD) accumulates less Na^+ and more K^+ than durum wheat (AABB) (Dubcovsky et al. 1996). Loci involved in salt tolerance have been identified on chromosomes 4H and 5H of barley, *Hordeum vulgare*, and 1Hch, 4Hch and 5Hch of *H. chilense* (Forster et al. 1990) thus showing that the gene pool of wild relatives may represent an important source of new loci for salt tolerance. A number of QTLs affecting salt tolerance were detected on 1H, 4H, 6H and 7H chromosomes.

8.4 Maize

8.4.1 Drought

Drought is one of the important abiotic stresses which results in significant yield losses in maize (*Zea mays* L.). It is necessary to overcome the drought effects in order to minimize yield losses. Although drought stress affects maize in almost all

growth stages, it is extremely sensitive in the period from 1 week before to 3 weeks after flowering (Banziger et al. 2000). Maize is widely regarded to be more susceptible to drought at flowering stage than other rainfed crops. This is due to a combination of several factors including physical separation of male and female flowers, floral asynchrony, non-receptivity of the silk, tassel blasting, trapped anthers and embryo abortion (Lu et al. 2011). Consequently, breeding maize for reproductive-stage drought tolerance could lead to the development of improved varieties able to withstand varying degree of water stress (Messmer et al. 2009; Zhu et al. 2011).

Tolerance to drought in maize is a polygenic trait and typically has low heritability and is characterized by high genotype x environment interaction (GEI). In the genetic improvement of maize, QTL, MAS and genetic engineering approaches are becoming the most useful tools to develop drought-resistant maize genotypes. Conventional breeding based on direct selection of phenotypes under drought has led to impressive yield gains in maize but underlying genetic causes largely remain unknown. QTL-based approaches can contribute significantly to the understanding of the genetic basis of crop performance, especially under drought-stress conditions and such knowledge may be crucial in designing cost-effective breeding approaches aimed at improving sustainability and stability of grain yield under adverse conditions (Collins et al. 2008).

8.4.2 QTL Mapping

Drought tolerance QTL studies in maize and other crops and the strategies for their use in MAS breeding programs have been extensively discussed in several comprehensive reviews (Araus et al. 2008; Collins et al. 2008; Tuberosa and Salvi 2009). QTL mapping for grain yield (GY) underwater stress and other associated traits such as anthesis silking interval (ASI) have been an active area of research especially in the past two decades. The QTL detected under water-stress and well-watered (WW) conditions can be categorized according to the stability of their effects across environmental conditions. A *constitutive* QTL is consistently detected across most environments, while an *adaptive* QTL is detected only in specific environment such as WS conditions (Collins et al. 2008). One of the earliest studies involving tropical germplasm under managed stress conditions identified 13 QTLs on chromosomes 1, 2, 4, 6, 7, 8 and 10 for grain yield, of which QTLs on chromosomes 1 and 10 were stable across WW and WS environments (Ribaut et al. 1997). Since then, a number of QTL regulating morpho-physiological component traits as well as GY have been reported in maize (Li et al. 2010; Messmer et al. 2009, 2011).

Although genetic dissection of drought tolerance in maize seems to have been widely reported, successful practical application of identified QTLs in maize improvement programs are scarce. The reasons are manifold, including genetic complexity, influence of genetic background, epistasis, profound QTL x environment interactions (QEI), population-specific nature of identified QTL and involvement of donor lines that are not agronomically elite (Collins et al. 2008; Truntzler et al. 2010).

8.4.3 QTLs of Drought Tolerance in Wheat and Barley

Most QTLs for drought tolerance in wheat, and its close relative barley, have been identified through yield and yield component measurements under water-limited conditions (Maccaferri et al. 2008; Mathews et al. 2008; McIntyre et al. 2009; Quarrie et al. 2006). Although yield is the most relevant trait to breeders, it is very difficult to describe accurately with respect to water use and to identify candidate regions for positional cloning. For this reason, very few studies have identified QTLs associated with specific components of drought response. Although the development of gene-based molecular markers and genome sequencing should accelerate positional cloning (Collins et al. 2008), the genomic regions associated with individual QTL are still very large and are presently unsuitable for screening in a breeding program.

The effect of drought on reproductive processes has been extensively described in cereals (Barnabas et al. 2008). Passioura (2007) suggested that floral infertility resulting from water deficit could be a promising target for improvement but no QTL studies for this trait have been published in wheat or barley. Improving the competence of the root systems to extract water from the soil also seems an obvious target for genetic analysis.

The identification of markers or genes associated with root growth and architecture would be particularly useful for breeding programs to improve root traits by molecular marker-assisted selection. Few papers have described work on the identification of QTLs for root traits in wheat. Ma et al. (2005) found a QTL for root growth rate under Al treatment. QTLs of root traits (primary/lateral root length and number, root dry matter) under control conditions and during nitrogen deficiency were identified in wheat (Laperche et al. 2006). However, QTLs corresponding to root architecture in dry environments are yet to be discovered in wheat and barley.

The genetic basis of drought tolerance has been studied extensively in maize and other taxa (Ribaut and Ragot 2007; Tuberosa et al. 2002). Over the past few years there have been several mapping studies that have targeted drought tolerance and other abiotic stress tolerance loci associated with performance in low-yielding environments. However, despite this substantial research effort the only markers that have found their way into practical plant breeding programs are those for boron and aluminium tolerance (Gupta et al. 2010).

8.5 Barley

Barley (*Hordeum vulgare* L.) is an important cereal crop in the Near East, Asia, Central Africa, North and South America, and Europe. It occupies more than 40 million ha in developing countries where often it is the only possible rainfed crop that farmers can grow (Ceccarelli 1994). It is an annual, diploid self-pollinating species with a relatively short life cycle. Its importance derives from the ability to grow and produce in marginal environments that are often characterized by drought and

low-temperature conditions. Among the cereals, barley is considered a good genetic model for the Triticeae tribe to study plant response to adverse environmental conditions. Its inbreeding behavior and diploidy make genetic studies easy to perform. It is a model species for genetic and physiological studies and shows a wide range of adaptations to various habitats; its wide range of adaptability, the availability of a wide range of genetic stocks, and the extended colinearity with other members of the tribe are additional advantages as a model (Hayes et al. 2003).

Molecular mapping of genes controlling plant resistance to drought stress has lagged behind that for disease resistance and morphological characters because of the complexity of drought tolerance and its association with many traits.

Molecular markers have played an important role in understanding the genetic basis of economically-important traits in barley. Over the last decade, comprehensive genetic maps have been constructed for seven barley chromosomes and have been used in QTL analysis (Kleinhofs and Graner 2001) and to isolate genes through map-based cloning (Kilian et al. 1995). An important use of markers has been MAS, which is made possible by the identification of markers linked to commercially-important traits such as disease resistance (Graner et al. 1996), response or tolerance to abiotic stress (Forster et al. 2000) and seed or feed quality traits.

Several approaches have been pursued to detect sequence polymorphisms in barley relying on hybridization RFLP-Restriction Fragment Length Polymorphisms (Graner et al. 1991) and PCR-based molecular marker systems like RAPD-Randomly Amplified Polymorphic DNA (Weyen et al. 1996), SSR-simple sequence repeats (Pillen et al. 2000; Ramsay et al. 2000; Thiel et al. 2003; Varshney et al. 2006), AFLP-Amplified fragment length polymorphisms (Waugh et al. 1997) and SNP-single nucleotide polymorphisms (Kota et al. 2001). However, RFLPs are technically complex, require large quantities of DNA and present a limitation to high throughput genetic analysis. More recently, trait-linked RFLP markers have been adapted for PCR amplification, making them amenable to MAS (Tacconi et al. 2006). Marker-assisted breeding is generally more efficient when molecular maps are saturated, due to an increased chance of finding polymorphic markers in any genetic background. To date, approximately 1,000 barley SSRs have been published (Pillen et al. 2000; Struss and Pliescke 1998), of which about one-half have been genetically mapped (Ramsay et al. 2000; Varshney et al. 2006; Wenzl et al. 2006).

8.5.1 Drought

Barley is characterized by immense variation with respect to drought adaptation and has been cultivated from boreal to equatorial regions of the world (Schulte et al. 2009). The slow progress in this area is mainly due to quantitative inheritance of drought-related traits. The advent of molecular tools has made it possible to dissect genetic inheritance of this trait-complex and several successful QTL analyses have been performed in barley and related species (El Soda et al. 2010; McKay et al.

2008; Teulat et al. 2003; Tondelli et al. 2006). These studies revealed that crop plants have evolved a number of drought-adaptive traits to maintain their life under water-deficit conditions.

8.5.2 Cold

In the Triticeae genome, the long arm of chromosome 5 has been the region most frequently reported to be associated with low-temperature tolerance and vernalization response (Francia et al. 2004; Vágújfalvi et al. 2003). Until recently, these genes have been reported as QTL effects because they show complex, rather than Mendelian inheritance. Most recently, two low temperature tolerance QTLs, approximately 25 cM apart, were reported in the winter x spring two-rowed barley population as Nure /Tremois (NxT) (Francia et al. 2004). The Nure/Tremois low-temperature tolerance Fr-H1 QTL corresponds to the Dicktoo/Morex (DxM) barley population LT tolerance QTL of Hayes et al. (1997), which in turn is coincident with VRN and flowering time (heading date) QTLs (Hayes et al. 1997). The NxT LT tolerance Fr-H2 QTL is syntenous with a LT tolerance QTL in diploid wheat (Vágújfalvi et al. 2003); COR gene product accumulation QTLs also map to this position in both species.

Recently, candidate genes have been mapped to these QTL positions. Barley HvBM5A maps to the VRN-H1 QTL position (Von Zitzewitz et al. 2005), in agreement with the position of TmAP1, the candidate VRN-Am1 gene of diploid wheat (Yan et al. 2003). It is possible that HvBM5A has pleiotropic effects on VRN response, flowering time and/or LT tolerance. However, Karsai et al. (2001) have found that these three traits occur in all possible combinations in barley, suggesting linkage, rather than pleiotropy, may be responsible for the expression. Beales et al. (2005) recently mapped the photoreceptor PhyC-a potential candidate for the day length-influenced flowering time QTL to this region in hexaploid wheat.

8.6 Sorghum and Pearl Millet

Sorghum is the world's fifth most important cereal crop and is the dietary staple of more than 500 million people spread in 30 countries. It is grown on 40 million ha in 105 countries of Africa, Asia, Oceania and the Americas. Africa and India account for the largest share (>70 %) of global sorghum area while USA, India, Mexico, Nigeria, Sudan and Ethiopia are the major sorghum producers (Ashok Kumar et al. 2011). Pearl millet (*Pennisetum glaucum* (L.) R. Br., a staple food crop grown under rainfed conditions on approximately 25 million ha of drought-prone arid and semi-arid regions in Africa and South Asia (FAO and ICRISAT 1996). Drought stress is a common feature in both sorghum and pearl millet and can occur any time during the crop cycle and terminal stress (flowering through grain filling) is more

damaging to the productivity of the crop than stress at the vegetative or preflowering reproductive stages (Mahalakshmi et al. 1987).

8.6.1 Drought

Sorghum is one of the most drought tolerant grain crops and its rich genetic diversity for stress tolerance makes it an excellent crop model and choice for studying the genetic and physiologic mechanisms of drought tolerance. However, water stress affects almost every developmental stage of the sorghum plant and damaging effects of this stress was more noted when it coincided with various growth stages such as germination; seedling shoot length, root length and flowering (Khayatnezhad et al. 2010; Rauf 2008).

Drought tolerance in sorghum is a complex trait affected by several interacting plant and environmental factors. Water loss from plant tissues under drought conditions results in growth inhibition and in a number of other metabolic and physiological changes including abscisic acid (ABA) accumulation, stomatal closure, decreased photosynthesis, increase in K^+ and Cl^- which *bind* water (Borrell et al. 2001). The growth stage at which moisture stress occurs is very important in determining the response or reaction of sorghum to water stress. Two distinct drought responses, namely pre-flowering and post-flowering drought responses, have been described in sorghum and are probably controlled by different genetic mechanisms (Rosenow 1987). Although somewhat difficult to combine, some sorghum hybrids containing both pre- and post-flowering drought tolerance have been developed (Rosenow et al. 1996).

Drought or any other abiotic stress results in marked reduction of yield and plant growth; as under inadequate water conditions, photosynthesis will be limited and consequently, limited availability of photosynthetic assimilates and energy to the plant, and plants use this limited supply of nutrients to their maximal advantage in order to survive under stress. Identification and understanding the mechanisms of drought tolerance in sorghum have been major goals of plant physiologists and breeders. The desirable traits include prolific root system, ability to maintain stomatal opening at low levels of leaf water potential, high osmotic adjustment and various seedling parameters (Rajendran et al. 2011) as under drought-stress conditions, an urgent need for plants would be to increase the uptake of water, which is usually more available deep in the soil (Xiong et al. 2006).

Water deficit is sensed by the roots which begin to synthesize ABA within one hour of the onset of the water stress. ABA is transported via xylem from roots to leaves within minutes to hours. Root length is an important trait against drought stress in plant varieties; in general, the variety with longer root growth has greater resistant to drought (Kaydan and Yagmur 2008; Leishman and Westoby 1994). Dhanda et al. (2004) reported that the osmotic membrane stability of the leaf seg-

ment was the most important trait, followed by root-to-shoot ratio and root length on the basis of their relationships with other traits for drought tolerance. These water sensitive stages may be exploited to discriminate genotypes on the basis of their resistance to water stress. Among these critical stages, water stress induced during the seedling stage has been exploited in various crop species to screen germplasm or breeders populations i.e. wheat (Dhanda et al. 2004), sorghum (Bibi et al. 2010; Gill et al. 2002), maize (Farsiani and Ghobadi 2009; Khayatnezhad et al. 2010) and sunflower (Rauf 2008). The excellent drought characteristics of sorghum make it one of the most important food and feed crops in the arid and semi-arid regions of the world.

8.6.2 QTL Mapping

Molecular markers have been used to identify and characterize QTLs associated with several different traits in sorghum, including plant height and maturity (Pereira and Lee 1995), characters concerned with plant domestication (Patterson et al. 1995), disease resistance (Gowda et al. 1995; Kebede et al. 2001), drought tolerance (Tuinstra et al. 1997, 1998) and heat, salinity and flooding (Ali et al. 2011; Ejeta and Knoll 2007). Several sorghum linkage maps have been established using RFLP and other DNA markers (Kong et al. 2000; Taramino et al. 1997) and more than 2,400 loci have been mapped on an inter-specific F_2 population of *Sorghum bicolor* × *S. propinquum* (Bowers et al. 2000). The average marker density of 0.5 cM or 350 kb between DNA markers is suitable for fine mapping of genes and QTLs. The high-density genetic map and its relatively small genome size make sorghum a suitable species for positional cloning.

In pearl millet, a number of genomic regions are associated with drought tolerance in terms of both grain yield and its components. For example, a QTL associated with grain yield per se and for the drought tolerance of grain yield mapped on linkage group 2, explained up to 23 % of the phenotypic variation. Some of these QTLs were common across stress environments whereas others were specific to only a particular stress environment. All the QTLs that contributed to increased drought tolerance did so either through better than average maintenance (compared to non-stress environments) of harvest index, or harvest index and biomass productivity. In addition, a number of genomic regions associated with traits that determine GY under stress have also been identified, but they were often not associated with either better maintenance of GY or with actual GY under stress. It was concluded that there is considerable potential for marker-assisted backcross transfer of selected QTLs to the elite parent of the mapping population and for their general use in the improvement of pearl millet productivity in water-limited environments (Yadav et al. 2002).

8.7 Conclusions and Prospects

Considering the importance of cereal crops as a predominant source of food around the world, identification of traits and genotypes associated with tolerance to stresses is vital for the crop improvement programs. The response to abiotic stresses such as drought, salinity, heat, cold and submergence are regulated by QTLs/genes/transcription factors etc., to provide stress tolerance in plants. Concerted efforts are required to fully understand the physiological and molecular basis of tolerance and emphasis should be on discovering potential candidate genes through evaluation of the genetic resources available followed by characterization, mapping and cloning. Functional analysis of these genes and transcription factors will thus provide more information on the intricate regulatory network pathways involved in abiotic stress responses and the cross-talk between different signaling pathways during stress adaptation. This will provide a clearer understanding of abiotic stress-related signal transduction events. The development of molecular genetic markers and the use of these markers in QTL analysis is increasingly becoming a common approach for accelerating gains from selection for complex quantitative traits in crop plants in breeding programs. However, lack of stability across different environments and QTL × E interaction evaluation remains a major impediment to the efficient use of MAS and further progress in the area can lead to a better understanding of the mechanisms involved and with the availability of precise markers, the transfer of the traits into desirable backgrounds will be quicker, easier and simpler. The identified QTLs, genes and transcription factors can also be genetically engineered to produce transgenic plants to confer higher levels of tolerance to various abiotic stresses. Further improvements in the area of QTLs and candidate genes/alleles will lead to development of crop varieties having superior levels of stress tolerance as crop improvement programs will have high precision.

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Chapter 9
Single Nucleotide Polymorphism (SNP) Marker for Abiotic Stress Tolerance in Crop Plants

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Abstract Agricultural crop production has been seriously hampered by various detrimental environmental conditions all over the world. Such conditions modify the growth and development of plants and ultimately reduce the economic yield enormously. These detrimental effects can be overcome by developing better stress-tolerance plants utilizing different genetic techniques. Therefore, there is a need to develop a marker system for the identification of stress responsive genes in order to combat the losses. Single nucleotide polymorphisms (SNPs) have become a more preferable marker over microsatellites because of their frequent occurrence in the genome and low rate of mutations. The discovery of SNPs in many crop species facilitates the availability and identification of many genes or quantitative trait loci (QTLs) associated with traits related to abiotic stress. Hence, identification of SNP flanking the genomic regions containing QTLs for aspects of abiotic stress tolerance

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would strongly expedite the targeted integration of this trait into another susceptible germplasm. Such identification of SNPs will not only promote marker-assisted breeding for abiotic stress tolerance but also open a vista for cloning and evaluation of primary genetic factors suitable for engineering improved abiotic stress tolerant plants. This review presents the present status of SNP marker technologies for abiotic stress tolerance in crop plants.

Keywords Abiotic stress • MAB • SNP • QTL

9.1 Introduction

The growth and yield of crop plants are highly influenced by abiotic stresses such as water deficit, high salinity and high and low temperature. It becomes a primary task to understand the underlying mechanisms for stress responses in plants to efficiently increase crop productivity under unfavorable or stressful conditions and the development of approaches for improving response of plants and adaptation to these stresses at the molecular, cellular, physiological and biochemical levels (Yamaguchi and Shinozaki 2006). The success of traditional breeding approaches for stress tolerance attempted so far utilized genetic differences resulting from varietal germplasm, distant hybridization, induced mutations and somaclonal variation from tissue culture plants have been limited. Those efforts produced only some plants possessing enhanced stress tolerance under field conditions (Flowers and Yeo 1995). Moreover, the complex nature of various abiotic stresses which are controlled by different genetic and environmental factors, limits breeding programs (Sinclair 2011). Modern agriculture has to implement new techniques to meet the food demands of an ever-growing world population as traditional crop improvement techniques reach their limits.

Molecular markers are extensively utilized in plant genetic research to speed up the plant selection process through marker-assisted selection (MAS) with special reference to particular traits or through the selection of chromosomal segments flanked by the markers at the genomic level (Collard and Mackill 2008). QTLs that are linked with tolerance at one developmental stage can vary from those linked to other stages (Foolad 1999). Furthermore, marker-assisted selection programs enable the direct integration of a particular gene of interest by genetic engineering and it appears to be a highly reliable and rapid method for improving stress tolerance.

Recently, SNP technology has attracted much interest among plant scientists that enables the identification and analysis of intraspecific sequence differences (Rafalski 2002). These are the most abundant type of genetic markers found in all organisms, where the variations are found at a single nucleotide position. SNPs techniques are particularly useful for genome-wide high throughput linkage and

also in the identification of association mapping, particularly of traits that are controlled by multiple genes (Jorde 1995, 2000).

In this chapter we discuss the recent advances in the discovery and implication of SNPs with special reference to major abiotic stress including drought, salinity and heat for the improvement of some important crop plants in order to mitigate the losses by abiotic stresses.

9.2 Single Nucleotide Polymorphism (SNP) of Haplotypes

SNP refers to a specific and defined position at a chromosomal site at which the DNA sequence of two genotypes differ by a single base; haplotype refers to the set of SNPs that correspond to a particular trait. These are naturally-occurring variants that affect a single nucleotide. SNPs are a second class of genetic markers that can be mined from sequence data and are useful for characterizing allelic variation, genome-wide mapping and as a tool for marker-assisted selection. Single nucleotide polymorphism discovery through sequencing therefore appears quite promising as a means to uncover variation in agriculturally-relevant populations (Robbins et al. 2011; Shirasawa et al. 2010). These are the most common type of sequence variation and tend to be biallelic in plant species (Ching et al. 2002). New methods for SNP detection are facilitating high-throughput genotyping, and provide strong motivation for the identification of sequence variation.

The genomes of many important crop plants have a large size, which is in some cases (e.g. barley, wheat and maize) are at least as large as or significantly larger than the human genome. Finally, a considerable number of crop plant species are not diploid but polyploids or ancestral polyploids. This makes SNP identification and evaluation much more difficult and complex than in a diploid organism such as in humans since SNPs between the different genomes have to be discriminated from SNPs between individuals (Durstewitz et al. 2010).

Next-generation sequencing (NGS) technologies have enabled the identification of large numbers of SNP markers in basically any crop plant via comparative sequencing of individuals (Varshney et al. 2009). In recent years, this process began in crop plants with the comparative sequencing of the transcriptome of different individuals after reverse transcription of messenger RNA (Barbazuk et al. 2007; Hasenmeyer et al. 2011; Hiremath et al. 2011; Novaes et al. 2008).

SNPs are less mutable as compared to other markers, particularly microsatellites. Although the biallelic nature of SNPs makes them less informative per locus examined than the multiallelic markers such RFLP and SSR (Xiong and Jin 1999), this limitation has been overcome by their abundance, which allows the use of a greater number of loci. SNPs can mark functionally important allelic differences and SNPs that flag individual alleles of known genes have been used widely as molecular markers (Nakitandwe et al. 2007).

9.3 SNP-Markers of Choice in Genomics and Breeding Research

SNPs being the most widely prevalent and common form of sequence differences in most of the organism genomes, they have higher potential for genotyping markers in comparison with the conventional markers like RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism) and SSR (simple sequence repeat). As the bio-technology sector advances, SNPs are rapidly becoming preferred genetic markers for use in marker-assisted breeding (Flint et al. 2003), map based cloning (Wang and Liu 2006), the study of evolutionary relationships between different species (Feltus et al. 2004; Hillier et al. 2007) and the identification of undesirable alleles linked to human diseases (Eberle et al. 2007).

At present, genomic research SNP has become the marker of choice due to the following useful properties: (a) SNPs are ample across the genomes; (b) huge pools of SNPs can be used to classify sets of polymorphic markers; (c) SNP markers are biallelic making allele employment more simple; (d) SNP data from various groups can be easily incorporated in a database and (e) SNP genotyping can be programmed, allowing for rapid, high-throughput marker genotyping. Additionally, SNPs are highly useful if they alter the activity of genes and the activity of the genes in stress response is determined and therefore linked to variations in plant performance and sorted out. The study of genetic variability for stress determining candidate gene sequences helps in determining an allele of the specific gene for abiotic stress (Roorkiwal et al. 2014). Such information can therefore be further used in breeding programs to develop better varieties using modern molecular-breeding approaches like marker assisted recurrent selection (MARS) or gene pyramiding.

In polyploid crop plants like potato, tobacco, cotton, canola and wheat, the identification of candidate SNPs is a very formidable task. Mostly, minor allele frequency can be employed as a tool to identify candidate SNPs in diploid species (Yu et al. 2011). However, in polyploid crops, because of the presence of either homoeologous loci from the individual subgenomes (homoeologous SNPs) or paralogous loci from duplicated regions of the genome polymorphic loci within a single genotype are found. These false positive SNPs cannot be employed in genetic mapping and they frequently cause a lower validation rate at the time of assays. A notable SNP validation in allopolyploids depends upon the differentiation of the sequence variation regions (Bus et al. 2012).

Although a SNP marker is a useful means for positional cloning, association study and evolutionary analysis, low SNP identification efficiency by allele-specific PCR (AS-PCR) still limits its employment as a molecular marker as in other markers like SSR. In order to get rid of this problem, primers with a single nucleotide artificial mismatch launched within the three bases closest to the 3′end (SNP site) have been employed in AS-PCR (Liu et al. 2012).

9.4 Mapping Genes/QTLs Using SNPs

SNPs are broadly used as markers in genetic mapping and QTL analyses. QTL marker databases for complex characters have become a daily tool in functional genomic research. QTL mapping is generally employed because of its simplicity and perception. In order to elevate the importance of SNP and QTL markers, different databases have been constructed (Kim et al. 2009).

Articulation of the high-density SNP chips with potential genotyping facilitates the genetic progress which can be obtained by genomic selection as compared to the conventional selection techniques. But, genotyping for all the selected candidates with high-density SNP may not be cost effective. A small subset of SNP identified from the high density SNP chip can be employed for identification of direct genomic breeding value (DGV) for each selected character (Solberg et al. 2008).

The SNP Database Network in Japan (http://snpnet.jst.go.jp/) gives an integrated account for SNP identification. The dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) gives a function for SNP identification across the whole plant genome. The BioMercator has been constructed to automatically detect map compilation and QTL meta-analysis, and also to observe associations between genes and QTLs through a graphical ally (Arcade et al. 2004). QTLNetwork is a software package for mapping the genetic makeup related to complex traits for experimental populations obtained from hybrids of two inbred lines (Yang et al. 2008). The Gramene QTL database (http://www.gramene.org/qtl/) provides QTLs related to various agronomic traits in rice, maize, barley and other crops.

9.5 Indels and SNPs for Abiotic Stress

Next-generation sequencing (NGS) technologies provide a new turn in the area of DNA sequencing and significantly improves genome sequencing. The identification of large numbers of sequence differences, such as SNPs and insertions/deletions (InDels), is one of the most important applications of NGS technologies (Huang et al. 2013; Varshney et al. 2009). These genetic differences among different population are the main criteria to determine the phenotypic differences, such as abiotic stress. SNPs have occupied remarkable importance because of their high abundance across the genomes, employed in identification of population structure and linkage disequilibrium analysis, capacity for high-throughput genotyping and cost effectiveness. In marker-assisted selection and fine mapping InDels have also been largely employed with great success (Hayashi et al. 2006; Liang et al. 2011). The location of SNPs and InDels within a genomic region could have altered both gene expression and function. DNA polymorphisms located within coding regions are important especially as they might modify protein function (Jain et al. 2014).

9.6 SNPs in Genomic Selection

Marker assisted selection (MAS) is particularly feasible in conditions, where the present selection accuracy is low, e.g. traits with low heritability. Genomic selection (GS) is used to obtain a high accuracy of selection. The basis of GS was first recommended by Meuwissen et al. (2001), where the markers that are in linkage disequilibrium with the particular gene affecting the trait are employed to determine direct genomic breeding value (DGV). In GS, markers are not employed in identification of a trait like in MAS. In GS high-density marker description is needed to potentially have all QTLs in LD with at least one marker. After that the compiled information on all possible loci, haplotypes and marker effects across the entire genome is employed for calculating the genomic estimated breeding value (GEBV) of a particular line in the breeding population.

The estimate of DGV is employed in genomic selection. It is calculated from the sum of SNP effects of single markers or haplotypes within a chromosomal region (Solberg et al. 2008). Such single genetic markers or haplotypes are adopted to explain most of the genetic variance contributed by the QTL (Hayes et al. 2009). Lack of dense marker arrays and the high cost linked with the genotyping of such markers have been the major limitations for the application of genomic selection (Meuwissen et al. 2001). At present, development in the field of molecular biology promotes the wide range employment of high density marker chips for selection of species and depends on their genomic usefulness. Generation and validation of numbers of markers have been possible due to fast-rate transformation of sequencing technologies and high-throughput (HTP) SNP genotyping schemes, providing a new vista for successful implication of GS in breeding for composite traits (Jannink et al. 2010; Mastrangelo et al. 2012; Resende et al. 2012; Zhao et al. 2012).

9.7 SNPs in PGRs for Abiotic Stress

SNPs which are considered as highly reliable DNA-based markers can easily detect differences between alleles in the plant genomic resources (PGRs). Therefore, it is useful in the study of genomic resources in various aspects. Compared to other markers, like SSRs, SNPs are widely prevalent and stable during inheritance, it thus provides an opportunity to study PGRs in various ways, such as the DNA fingerprinting of cultivar, generation of genetic maps, study of genetic diversity, identification of association between genotype and phenotype and marker-assisted selection (Ganal et al. 2009). Large numbers of SNPs have been identified in many crops from the various available sequence information (Table 9.1). Conventionally, the sequence variations are compared among the large number of PGRs comprising diverse genetic background. For example, in the identification of large numbers of SNPs in grapevine (Lijavetzky et al. 2007) and black cottonwood (*Populus trichocarpa*) (Geraldes et al. 2011), ESTs as well as transcriptomic sequences were employed. In wheat, multi-alignments of reserved domains in DREB1, WRKY1

Table 9.1 Distribution of SNP for major abiotic stress in different crops

Crops	Type of abiotic stress	Remarks	References
Maize (*Zea mays*)	Drought	SNPs in genes involve in modification of abscisic acid levels	Setter et al. (2011)
Maize (*Z. mays*)	Drought	SNP mutations in MYBE1 transcription factor gene	Assenov et al. (2013)
Wheat (*Triticum aestivum*)	Drought	SNPs in TaDREB1 transcription factor	Chen et al. (2005)
Rice (*Oryza sativa*)	Drought	SNPs in the regulatory region of ERF3, a candidate gene for drought tolerance through EcoTilling	McNally et al. (2009)
Barley (*Hordeum vulgare*)	Drought	SNPs related to starch generation/ degeneration genes during acute drought period	Sebastian et al. (2011)
Field pea (*Pisum sativum*)	Salinity	SNP technology and related genetic linkage maps favored recognition of QTLs and candidate genes for salinity tolerance	Leonforte et al. (2013)
Rice (*O. sativa*)	Salinity	SNPs from 21 clones of 20 salt stress responsive genes in 23 rice mutant lines from sequence variations analysis in both the exon and intron region	Hyun et al. (2011)
Rice (*O. sativa*)	Salinity	SNPs linked to salt-associated genes, engaged in mechanisms like Na^+/K^+ ratio equilibrium, signaling pathway and stress preservation from the association studies	Sonia et al. (2013)
Wheat (*T. aestivum*)	Heat	The first report of SNP marker in the HSP16.9 gene of bread wheat was made to identify heat tolerant and heat susceptible genotypes using an allele-specific PCR primer	Garg et al. (2012)
Rice (*O. sativa*)	Heat	SNPs linked with thermo tolerance in the BC1F1 and F_2 populations obtained from IR64 × N22 cross population adopting selective genotyping and single marker analysis	Ye et al. (2012)
Cowpea (*Vigna unguiculata*)	Heat	Haplotypes associated with QTLs for heat tolerance	Lucas et al. (2013)

transcription factors (TFs) and HKT-1 had been employed to construct specific primers for the detection of functional SNPs. Different genotypes of durum wheat that have different tolerance ability to salt and drought stress were used to validate these primers (Mondini et al. 2012). Similarly, several genic SNPs linked to tolerance to various abiotic stress such as cold tolerance in barley (Tondelli et al. 2006), frost tolerance in rye (Li et al. 2011), drought tolerance in maize (Hao et al. 2011; Lu et al. 2010) as well as in *Arabidopsis* (Hao et al. 2004, 2008) have been identified. Such stress-specific SNPs could be transformed into functional markers to utilize in particular crop improvement strategies by marker-assisted selection.

9.8 SNPs for Major Abiotic Stress in Crop Plants

9.8.1 Drought

Drought is one of the most detrimental abiotic stresses in the world and it greatly reduces crop production and productivity. Although different techniques have been utilized to mitigate the problem of drought, breeding techniques through traditional breeding or genetic engineering become a proficient way for designing crops able to grow effectively in drought-affected areas. In spite of remarkable progress made by plant breeders through conventional breeding in generating drought tolerant varieties, the techniques are highly time-consuming, and labor and cost exhaustive. On the other hand, MAB is a more dynamic method, capable of utilizing many genomic regions of a crop under stressful environments, which were absolutely impossible by any previous approach. QTLs related to drought tolerance have been pointed out in various crop species. The construction of high resolution molecular genetic maps and marker-assisted selection technology have made possible the pyramiding of desirable drought tolerance genes (Ashraf 2010).

With the discovery of gene-based different SNP markers, the limited applications of molecular markers which existed before have been overcome. Employing different SNP markers, construction of high resolution linkage maps is possible for the identification and depiction of genes related to drought tolerance. Furthermore, the availability of genome sequence information of different crops, incorporation of genetic and physical maps and SNP markers for particular traits will accelerate the pace of molecular breeding for drought tolerance. The first effort to clone QTLs for drought tolerance was made by Salvi and Tuberosa (2005), and is considered a turning point in molecular breeding, have played a crucial role in better interpretation and exploitation of traits related to drought tolerance (Cattivelli et al. 2008; Tondelli et al. 2006; Tuberosa and Salvi 2006.

Drought tolerance is controlled by multiple genes; therefore, a candidate gene approach helps in dissecting the multiple traits. Association mapping based on candidate genes for drought tolerance characters was performed among 192 different entries of perennial ryegrass (*Lolium perenne* L.) collected from 43 countries. A collection of candidate genes related to antioxidant metabolism, dehydration, flow of water across membranes and signal transduction, were accomplished from complete 2,520 expressed sequence readings, against which 346 SNPs were recognized. During dry spell condition, coalitions between the gene for late embryogenesis LpLEA3 and LpFeSOD inscribing iron superoxide dismutase and leaf water content were reported. The association of over and above between genes for cytosolic copper-zinc superoxide dismutase and chlorophyll fluorescence, LpCyt Cu-ZnSOD, under drought conditions, were also indicated. Out of these established four remarkably associated single nucleotide polymorphisms; three were transposed to amino acid substitutions in various genotypes. The study depicted that differences between alleles of these genes could alter the response of the entire plant of perennial ryegrass to drought stress (Xiaoqing et al. 2013).

In maize (*Zea mays* L.), association mapping with candidate genes helps in identification of SNPs in genes involved in modification of abscisic acid levels in floral tissue during water-limited conditions (Setter et al. 2011).

Identification of SNP mutations in MYBE1 transcription factor gene, related to drought stress tolerance, of 26 tolerant and sensitive maize inbred lines from the gene banks of Maize Research Institute Zemun Polje, Serbia and Maize Research Institute, Kneja, Bulgaria, were carried out by employing direct PCR sequencing techniques. Although the detected mutations were not found in the conserved R1 domain of the MYBE1 protein, one of them was observed in the Ser/Thr-rich area. A particular SNP was observed only in different drought tolerant Serbian inbreds T3, T5, T6, T7 and T8 which was not found in the Bulgarian maize inbreeds. Detection of SNP mutations accompanied with genes related to drought stress tolerance in maize will help in the development of functional markers for efficient use in marker-assisted breeding for drought tolerance (Assenov et al. 2013).

TaDREB1 is an important transcription factor related to drought stress. Therefore, a study was conducted for the identification of SNPs in the TaDREB1 gene based on sequencing on 20 hexaploid cultivars and 3 diploid species of wheat. Out of 38,038 bp nucleotide sequences of TaDREB1, 271 SNPs and 14 InDels (insertion and deletion) were identified, on average of 1 SNP and 1 InDel for every 140 and 2,717 bp, respectively. The nucleotide diversity value in the hexaploid ($\pi = 0.01029$) was found to be lower than the diploid ($\pi = 0.02188$) indicating the high diversity of the TaDREB1 gene in the diploid rather than the hexaploid due to stronger selection pressure of the hexaploid than the diploid. From the haplotype analysis it was observed that SNPs in TaDREB1 were correlated with a drought-tolerant trait. Moreover, two haplotypes, one for drought tolerant accession and one for drought susceptible accession, were also detected (Chen et al. 2005).

The technique of EcoTILLING was employed in 900 *Oryza sativa* lines for 1,800 bp of coding and regulatory region of ERF3, a candidate gene for drought tolerance and identified 31 SNPs and short indels that grouped into nine haplotypes from the sequence information of selected lines (McNally et al. 2009).

The narrow genetic bases of modern crop cultivars provide a great barrier in the breeding of cultivars that could perform well in the present scenario of climate change. So, it is high time to give more importance in investigating the concerned allelic diversity simultaneously in modern cultivated crops and their wild relatives. In recent years the illustration of SNPs on the linkage map of barley has gained momentum (Close et al. 2009; Sato et al. 2009; Stein et al. 2007), and recently a SNP-based map showing different gene sequences responsible for various abiotic stresses has been established (Rostoks et al. 2005). The investigation based upon the identification of genetic diversity among barley genotypes, in order to intensify its response to terminal drought stress during plant growth and development stage and the expression patterns of drought regulated genes, were examined, mapped and the position of these genes was integrated into a complete SNP linkage map of barley. Haplotypes confined in a stock of 17 starch generation/degeneration genes were described, and within the genes related to sucrose synthase (types I and II) and starch synthase specifically, a large amount of haplotype variation was discovered.

The capability of 50 barley genotypes to preserve grain starch content during extreme drought situations was analyzed. Thus, the assembled idea denotes a useful source for the generation of functional markers to determine a large number of barley genotypes to determine related haplotypes of starch generation/degeneration genes to seed starch content during drought and, therefore, will promote better development of barley genotypes for improved grain weight (Sebastian et al. 2011).

9.8.2 Salinity

Soil salinity is a severe abiotic stress limiting plant growth and crop production globally (Ondrasek et al. 2011). The application of molecular markers in MAS for physiologically complex traits like salinity tolerance has barely been accomplished (Ashraf and Foolad 2013). Under this situation, breeders should emphasize selection of flexible and diverse genomic regions or reaction systems established in diverse genotypes, diverse screening surroundings and in distinct growth period. Hence, there is the necessity for estimating the adaptive make up (Collins et al. 2008) of various QTLs for varying salinity stress.

SNP markers accompanied with expressed sequence tags (ESTs) were established and utilized for the generation of exhaustive linkage maps for field pea. A complete 705 SNPs (91.7 %) were favorably identified and the sequences linked with the mapped molecular markers were employed for comparative genomic analysis among other legume species. SNP markers relevant for selection of salinity tolerance cultivars were flanked with the QTLs observed on linkage groups Ps III and VII. Genomic regions containing candidate genes associated with saline stress tolerance supported by these SNP markers were observed in the genome of the legume *Medicago truncatula* Gaertn., derived from the sequence comparison. The SNP technology and related genetic linkage maps developed favored recognition of QTLs and candidate genes for salinity tolerance (Leonforte et al. 2013).

A total of 516 SNPs from 21 clones of 20 salt stress responsive genes in 23 rice mutant lines and their original variety Dongan were generated from sequence variations analysis. A total of 90 SNPs were developed by plasma membrane ATPase 1 and 110 SNPs were identified in potassium transporter 12. ATPase/ABC transporter again developed 75 SNPs. Peptidyl-prolyl cis-trans isomerase, which has different functions in plants like calcium regulated signaling processes, protein folding, wound and temperature stress, developed the highest number of SNPs (134). SNPs together in exon and intron regions were shown by five clones and nine clones developed SNPs either in exon or intron. Differences were highly developed by DM4 and DM5 compared to other lines and significantly diverse haplotypes. From the total of 516 SNPs, only eight developed significance characterized by p-value (<0.05) and $R^2(>0.1)$. Although such SNPs were identified in the intron region, there is potential to classify them as a marker for discovering salt tolerant characters (Hyun et al. 2011).

For the identification of genotypic variation pertaining to salt stress, 392 rice accessions were genotyped by the EcoTILLING approach. Five chief salt-associated genes, engaged in mechanisms like Na^+/K^+ ratio equilibrium, signaling pathway and stress preservation, were aimed and discovered 40 novel allelic variants in the coding regions. Eleven representative SNPs linked to salinity were discovered from the association studies employing general and mixed linear models together. Out of five non-synonymous SNPs representatively linked to salt-stress characters, a T67K mutation which is supposing to diminish one transmembrane domain in OsHKT1; and a P140A transition that highly elevate the possibility of OsHKT1; five phosphorylation were identified. The mutation in K24E could presumably modify synergistic action of salt with other relating proteins thus influencing its function. The study revealed allelic differences influencing salinity tolerance which could be a fundamental tool for developing a salt-tolerant variety (Sonia et al. 2013).

9.8.3 Heat

An increase in global temperature poses a grave threat to agricultural crop production. The Intergovernmental Panel on Climate Change has predicted an increase of 2–4.0 °C in global temperature by the end of the current century (IPCC 2007a, b). The detrimental effects of heat stress can be overcome by breeding heat-tolerant crop plants employing different genetic techniques. Genetic techniques to discover and trace QTLs related to heat tolerance allow marker-assisted breeding for heat tolerance, as well as opening new vistas for cloning and evaluation of related genetic causes which will be helpful in developing improved heat-tolerant plants (Wahid et al. 2007).

Tolerance to heat stress is a complex phenomenon, controlled by multiple genes imparting a number of physiological and biochemical changes. The first report of a SNP marker in the HSP16.9 gene of bread wheat was made to identify heat tolerant and heat susceptible genotypes using an allele-specific PCR primer. DNA fragments covering a partial sequence of wheat (*Triticum aestivum* L.) HSP16.9, were amplified from a heat-tolerant genotype (K7903) and heat-susceptible genotype (RAJ4014), and subsequently analyzed for the presence of the SNP. One SNP was found between these genotypes and the analysis of amino acid sequence showed that the base transition (A/G) positioned at 31 amino acid resulted in missense mutation from aspartic acid to aspargine residue. Allele specific primers based on SNP were designed to screen the other heat tolerant and susceptible genotypes. On the basis of a heat sensitivity index (HSI) for grain yield, out of 18 genotypes, 12 were categorized as tolerant and the rest as susceptible. The SNP marker identified 10 of the 12 tolerant genotypes. SNP marker associated with terminal heat stress in wheat may serve as an informative molecular marker that can be used to improve heat tolerance in wheat (Garg et al. 2012).

In order to determine QTLs related to heat tolerance in rice, the progeny of BC_1F_1 and F_2 populations obtained from IR64 × N22 cross were treated at 38/24 °C for

14 days at the flowering stage, and spikelet fertility was evaluated. With the employment of 384-plex Illumina GoldenGate genotyping assay the F_2 and selected BC_1F_1 plants were genotyped and four single nucleotide polymorphisms were found to be linked with heat tolerance in the BC1F1 population adopting selective genotyping and single marker analysis, and four representative QTLs for heat tolerance in the F2 population. It was also found that two major QTLs were identified on chromosome 1 (qHTSF1.1) and chromosome 4 (qHTSF4.1) and they denote 12.6 % (qHTSF1.1) and 17.6 % (qHTSF4.1) of difference in spikelet fertility at high temperature. The influence of qHTSF4.1 on chromosome 4 was validated in the selected BC_2F_2 progeny of IR64 × N22 cross, and the plants having qHTSF4.1 displayed considerably higher spikelet fertility compared to others (Ye et al. 2012).

QTL analysis employing 141 genotypes from a recombinant inbred population developed from a cross between CB27 and IT82E-18 cowpea varieties has been implemented to provide reserves for breeding improved a heat-tolerant cowpea variety. A range of 11.5–18.1 % of the phenotypic differences were interpreted from the five domains exhibiting 9 % of the cowpea genome and labeled with 48 transcript-obtained single nucleotide polymorphism markers. The parent CB27 provided appropriate haplotypes specifically for the four QTLs, on the other hand IT82E-18 for the fifth QTL was the reserve for heat tolerance (Lucas et al. 2013).

9.9 Conclusions and Prospects

At present, large-scale identification of SNPs for abiotic stress tolerance in crop plants is still a challenge whether the whole genome or only the coding sequences are examined for SNP identification. A new technique called sequence capture which has been successfully used for SNP detection in exons of the human genome (Hodges et al. 2007) may also be employed in crop plants. In this technique, exon sequences are assayed against an array and hybridized with total genomic DNA and further sequenced by any of the next-generation sequencing technologies. Large numbers of productive SNPs are believed to be predicted quickly by reference to the array sequences. On the other hand, these methods have not yet been utilized in crop plants, and there is confusion regarding the authenticity of this method for identification of SNPs in orthologous sequences after all paralogous sequences are also identified by this method. If the multiplex amplification technique (Krishnakumar et al. 2008; Porreca et al. 2007) based on sequenced amplicons associated with barcoding approaches for the sequencing of particular fragments are used at the same time from various lines (Martin et al. 2009), there is the hope to implement SNP discovery for abiotic stress related traits in a variety of crop plant, utilizing the next-generation sequencing methods, in a quick and economical way.

Moreover, genetic engineering techniques for developing stress tolerance are at an early stage and the achievements to date are just a starting point. Amelioration of SNP marker in genetic research will accelerate greatly for the development of plants responsive to abiotic stress in future. The available marker-assisted technology pro-

vides a possible means to deport multiple genes that may collaboratively promote abiotic stress tolerance in plants. Compiling further information on other stress tolerance factors and the identification and cloning of related genes will accelerate introgression of different genes and generation of high-stress tolerant plants. Hence, extra effort is greatly required to obtain a fair knowledge of the genetics, biochemical and physiological mechanisms related to abiotic stress tolerance in crop plants.

Single nucleotide polymorphisms represent an important plentiful origin of polymorphic markers which is useful for developing the high-resolution genetic maps of complex traits, and also for association studies based upon candidate gene approaches. Progress towards the identification of SNPs in major crop plants can be elevated when it is engaged with next-generation sequencing methods. In spite of obtaining the full genomic sequence of major crop plants available in the near future, large numbers of SNPs can be identified in diverse genes when sequence capture techniques are employed in association with next-generation sequencing technologies. SNPs haplotypes found in the genome of many crop plants will open a new indication favoring the thorough study of germplasm, dynamic association analysis of SNP markers with trait of interest and, finally, the efficient exploitation of genetic diversity on a whole plant genome level.

Additionally, to gain excellent knowledge about the molecular basis of plant response to abiotic stress along with tolerance, employment of genomics, proteomics and transcriptomics techniques are essential. The principles behind the molecular mechanisms of abiotic stress tolerance will provide the means for engineering abiotic stress tolerant plants and perhaps the basis for crop production which can perform well and yield economically under stress environments.

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Chapter 10
Transgenic Approaches for Abiotic Stress Tolerance in Crop Plants

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Abstract Abiotic stresses including drought, salinity and cold are a major challenge for sustainable food production as they may decrease the potential yields in crop plants by 70 %. Success in breeding for better adapted varieties to abiotic stresses depends upon intensive efforts using novel biotechnological approaches, including molecular biology, genetics, plant and cell physiology and breeding. Many abiotic stress-induced genes have been identified and some have been cloned. The use of current molecular biology tools to reveal the control mechanisms of abiotic-stress tolerance, and for engineering stress-tolerant crops is based on the expression of specific stress-related genes. Hence, plant genetic engineering and molecular-marker approaches allow development of abiotic stress-tolerant germplasm. Transgenic plants carrying genes for abiotic stress tolerance are being developed, mainly by using *Agrobacterium* and biolistic methods; transgenics carrying different genes relating to abiotic stress tolerance have been developed in crop plants like rice, wheat, maize, sugarcane, tobacco, *Arabidopsis*, groundnut, tomato and potato. This chapter

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focuses on recent progress in using transgenic technology for the improvement of abiotic-stress tolerance in plants. It includes discussion of metabolic engineering for biosynthesis and accumulation of compatible osmolytes (i.e. proline, glycine betaine, ectoine and polyols), reactive oxygen species formation under abiotic stress, ROS scavenging and detoxification in plant cells, single gene transgenic versus multiple genes and transcription factors and their roles in management of abiotic stresses.

Keywords Abiotic stress • Transcription factors • Osmoprotectants • Reactive oxygen species • Proline • Glycine betaine • Drought • Salinity

10.1 Introduction

Globally, agriculture uses approximately 70 % of the available fresh water and irrigation accounts for up to 90 % of total water withdrawals in arid nations, particularly in the developing countries of Asia and Africa (FAO 2009a; World Water Council 2008). Approximately 40 % of all crops in developing countries are grown on irrigated land, which account for only 20 % of the total arable land in these nations (FAO 2009b). The water withdrawal requirements for irrigation is expected to increase by 14 % in developing countries by 2030 and strategies to reduce this demand by developing crops requiring less irrigation, play a vital role in maintaining the world's food supply.

The growth and productivity of crop plants are adversely affected by abiotic stresses such as drought, extreme temperatures and high salinity. It has been estimated that approximately 70 % of worldwide agricultural yield reduction is due to the direct affect of abiotic stresses (Acquaah 2007). In the event of crops experiencing any abiotic stress a number of changes at the morphological, physiological, biochemical and molecular levels are triggered. Stress-induced gene expression can be broadly categorized into three groups: (1) genes encoding proteins with known enzymatic or structural functions, (2) proteins with as yet unknown functions and (3) regulatory proteins (Bhatnagar-Mathur et al. 2008).

The transgenic approach offers a powerful means of incorporating a broad spectrum of genes with profound ability to up- or down-regulate specific metabolic steps associated with different abiotic stress response. As abiotic-stress tolerance is a complex polygenic trait, although genetic transformation is considered difficult, results obtained from transfer of a single gene that encodes either biochemical pathways or the endpoint of signaling pathways, have been encouraging. In crop plants, various examples of transgenics have been reported to synthesize and accumulate osmoprotectants and molecular chaperons, reactive oxygen scavenging/detoxification enzymes, late-embryo genesis abundant protein genes, ion/proton transporters and water channels/aquaporins. These reports indicate that there has been a marginal to significant increase in drought and salt tolerance. Plants engineered to synthesize and accumulate osmolytes such as mannitol, sorbitol, glucose, fructose, ononitol, proline, glycine and betaine have shown marginal improved tolerance against abiotic stresses (Sakamoto et al. 1998).

Among the other abiotic stresses, drought is the major significant environmental stress that severely impacts the plant growth, development, yield, membrane integrity, pigment content, water relations, photosynthetic activity (Benjamin and Nielsen 2006) and performance than any other biotic factor (Shao et al. 2009). In the face of increased water shortages and yield losses due to water scarcity, it is essential for breeders to develop drought tolerant high yielding genotypes. Therefore, tremendous effort is being made to enhance the crop yield in the face of drought stress by both conventional and molecular breeding (MB) techniques. Because the progress of improvement through conventional breeding has met with limited success due to the complex polygenic nature of drought tolerance, molecular breeding has been exploited in this regard (Khan 2012).

Signal perception is the first step of the plant response to abiotic stress in which a sensor can perceive environmental stimuli and transmit signals to cellular targets. The two conserved proteins, namely response regulator (RR) protein and histidine protein kinase, play an important role in receiving and transducing intracellular and extracellular signals in a plant. The activated histidine kinase, transfers the phosphorylated group to an aspartate residue in the (RR) protein, resulting in an activation of downstream signaling cascade (Tena et al. 2001). Once an extracellular stimulus is perceived, second messenger molecules like Ca^{2+}, reactive oxygen species (ROS) and inositol phosphates participate in stress adaptation by activating a downstream signal cascade that affects phosphorylates transcription factors and protein kinases, which play a vital role in signal transduction mechanism (Xiong et al. 2002). CBL-CIPK, CDPK and MAPK pathways have been reported to be involved in stress signaling in plants. In addition, ABA signaling and transduction activates SnRK2s phosphorylate and subsequently induce expression of AREB/ABF/ABI5 target genes (Chae et al. 2007). The overall molecular process of signal transduction from the perception of the stresses to gene expression involves the two groups viz. proteins that function in protecting cells from dehydration (functional proteins) and second group contains protein factors that involve in regulation of gene expression and stress tolerance (Shinozaki et al. 2003). From the molecular point of view, the drought tolerance mechanism involves major functional and regulatory proteins. Functional proteins are the enzymes associated with the synthesis of osmotically-active compounds such as transporters, chaperones, ROS (reactive oxygen species) scavengers, osmolytes like fructan, trehalose, proline, glycine betaine, mannitol and polyamines, and protective proteins such as LEA and heat-shock proteins.

Molecular breeding strategies for improving crop yields against abiotic stresses involve biotechnological applications in three broad disciplines: (1) DNA marker technology for enhancing selection efficiency and precision in breeding; (2) genetic engineering (GE) for transferring agronomically useful traits across species without any barrier and (3) genomic tools for identifying new and useful genes/alleles. Among these molecular tools, genetic engineering or genetically-modified crop technology permits scientists to mobilize useful genes from a totally unrelated gene pool into crop plants without any barrier and with the least disruption to plant genome. Gene modification (GM) or gene technology is often proposed as a solution for increasing crop yields worldwide, particularly in less-developed areas that

are threatened by food insecurity and low crop productivity (Nelson et al. 2007). Transgenic approaches are being widely used throughout the world for abiotic stress tolerance in various crops (Ashraf 2010). Recent studies have reported the development of transgenic plants with various genes that encode several transcription factors (TFs), heat shock proteins (Hsp), late embryogenesis abundant proteins (LEA) and compatible organic osmolytes.

Transcription factors (activators and repressors) and cis-acting promoter elements are critical regulators of the changes in gene expression during drought-tress responses and their overexpression has been proved to be useful for improving stress tolerance in plants (Shinozaki et al. 2003). Extensive research in genomics coupled with bioinformatics and stress biology have provided the identification of useful genes for conferring abiotic stress tolerance and such superior genes are being transferred into elite genotypes through integrated MB and GE approaches (Varshney et al. 2011). The accessibility of whole genome sequences together with efficient and high-throughput techniques has facilitated the unravelling of countless genes related with abiotic stress. Bioinformatics tools have speeded up the process of identification of stress-associated gene families across species based on homology and gene synteny. In addition to whole genome sequencing, cDNA libraries related to stress tolerance, and genome-wide association studies have resulted in the discovery of abiotic stress-related genes (Kumari et al. 2014).

The objective of this review is to highlight the latest developments in the field of genetic engineering for development of abiotic stress tolerant crop plants. The different approaches being used and the various types of genes cloned and transferred in crop plants for conferring abiotic stress tolerance will also be discussed. Also included is discussion on the metabolic engineering for biosynthesis and accumulation of compatible osmolytes (i.e. proline, glycine betaine, ectoine, polyols), reactive oxygen species formation under abiotic stress, ROS scavenging and detoxification in plant cells, single gene transgenic vs multiple genes and transcription factors and their roles in management of abiotic stresses.

10.2 Abiotic Stress in Crop Plants

Owing to their sessile nature, plants are continuously exposed to a broad range of environmental stresses (Suzuki et al. 2014). Among various abiotic stresses, water availability (drought, water logging), ion-toxicities (salinity, heavy metals) and nutrient deficiency/imbalance, extreme temperatures (heat, cold, chilling, freezing), high light intensity and UV radiation hold significance and have devastating impacts on plant growth and yield besides causing deterioration of the quality of plant product. Often, combinations of two or more such stresses occur simultaneously and in such cases the severity is usually aggravated. Although it is a difficult task to accurately estimate the effects of abiotic stress on crop production; however, it is evident that abiotic stress continues to have a significant impact on plants as revealed by the percentage of land area affected as well as the number of publications directed at

various abiotic stresses (Cramer et al. 2011). It was reported on the basis of an FAO report published in 2007 (http://www.fao.org/docrep/010/a1075e/a1075e00.htm) that around 96.5 % of global rural land area is affected by abiotic stress, whereas more than 35,000 publications (2001–2011) dealt directly with abiotic stress (Cramer et al. 2011). Since world population is increasing at an alarming rate, and therefore minimizing the crop loss due to abiotic stresses coupled with increasing the crop productivity has become the highest agricultural priority (Tuteja et al. 2011). Genetic engineering has shown creditable success in production of transgenic plants with enhanced tolerance against abiotic stresses and is seen as a long-term solution for producing high-yielding transgenic crops on affected land areas around the globe.

10.3 Plant Transformation Approaches

The success of transgenic research necessitates effective plant transformation methods for stable integration and functional expression of foreign genes into the plant genome. Since the initial reports in tobacco (De Block et al. 1984; Horsch et al. 1984; Paszkowski et al. 1984), rapid developments in transformation technology have resulted in the genetic modification of a large number of plant species (Narusaka et al. 2012).

Overall, two types of methods are available for transferring gene(s) to plants, one mediated by a biological vector like *Agrobacterium* and another, direct gene transfer approach, where DNA is introduced into the cells by physical, chemical or even electrical means. Direct or non-biological gene transfer methods include particle bombardment, DNA uptake into the protoplasts in the presence of polyvalent cations, protoplast fusion with bacterial spheoplasts and with liposomes containing foreign DNA (lipofection), electro-transfaction, polymer-based transfection (polyfection), silicon carbide fiber-mediated DNA uptake, injection based methods (micro- and macro-injection), wave and beam mediated transformation, desiccation-based transformation, and exogenous DNA application and imbibitions (Darbani et al. 2008; Narusaka et al. 2012; Tsaftaris et al. 2000). The use of biolistics or particle bombardment is by far the most widely used direct gene transfer methods.

Although direct gene transfer methods are useful for stable transformation, as well as transient expression, there are still some issues related to low frequency of stable transformation, unwanted genetic rearrangements due to high copy number of genes and the longer period required to regenerate whole transgenic plants. Therefore, with extensive and increasing host range, *Agrobacterium tumefaciens* is the method of choice for gene transfer to all major crop plants. This soil bacterium possesses the natural abilities to deliver a well-defined part of its plasmid DNA (transfer DNA or T-DNA) into the nuclear genome of its host plant. The T-DNA integration and expression of its encoded native bacterial genes cause a neoplastic growth and produce tumors on infected plants, indicating a successful genetic transformation event (Chung et al. 2006). The bacterial genes are replaced with gene(s)

of interest, and interestingly it does not affect the transformation process or frequency, and therefore has been the molecular basis for *Agrobacterium*-mediated genetic transformation protocols (Gelvin 2003; Lorence and Verpoorte 2004).

Through extensive efforts made by the scientific community, remarkable progress has been witnessed during last three decades in plant genetic engineering. The optimized protocols have been developed for *Agrobacterium*-mediated genetic transformation of wide range of crop plants including monocots previously considered outside the *Agrobacterium* host range. Recently, success has been achieved in developing in planta transformation methods. Inspired by the complex patent landscape of *Agrobacterium* technology and in search of a utopian (open source) platform for plant biotechnology, Broothaerts et al. (2005) have identified three non-*Agrobacterium* species namely *Rhizobium* sp. NGR234, *Sinorhizobium meliloti* and *Mesorhizobium loti* as capable of successful genetic transformation of different plant species. A recent addition to this list of biological vectors for genetic transformation of plants is the virus-based vectors.

10.4 Metabolic Engineering for Biosynthesis and Accumulation of Compatible Osmolytes

Stimulated biosynthesis and accumulation of low molecular weight organic metabolites, collectively known as compatible osmolytes (as they do not inhibit the normal cellular functions) is considered as the most effective of the mechanisms evolved by plants to maintain their integrity and survival under multiple abiotic stresses. These chemically-diverse organic compounds figure among the most fundamental solutes in living organisms, being present from bacteria and fungi to higher plants and animals. They may fall into a few categories such as amino acids (proline, glutamate, glutamine, alanine) and their derivatives (ectoine, hydroxyectoine), quaternary amines (glycine betaine, polyamines, dimethyl sulfoniopropionate, DMSP), sugars (trehalose, sucrose), and polyols including sugar alcohols (mannitol, sorbitol, galactinol) (Jewell et al. 2010; Khan et al. 2009). These small, hydrophilic, usually uncharged or zwitterionic molecules get amassed in cytoplasm and remain non-toxic even at molar concentrations. They perform a vast array of functions including acting as reactive oxygen species scavengers, cell redox balancers, osmoprotectors or osmoticums, stabilizers of cytosolic pH, proteins, enzymes and membranes besides being a source of carbon and nitrogen during stress conditions and recovery phases. Owing to their multifunctions, osmolyte-mediated osmoadaptation strategy is widespread in nature, not only in plants but also in bacteria, fungi, animal and human cells (Burg and Ferraris 2008; Pastor et al. 2010; Yancey 2005). A strong correlation has been reported on several occasions between the enhanced synthesis and accumulation of these adaptor molecules and better plant abiotic-stress tolerance. An important feature of these osmolytes is that their beneficial effects are largely not species specific and therefore alien osmoprotectants can be introduced into plants to their new host (Kathuria et al. 2009). It is widely

believed that osmoregulation is the best strategy for abiotic stress tolerance, especially if osmoregulatory genes could be triggered in response to drought, salinity and high/low temperatures.

However, among these compatible osmolytes, some are widespread in plants (proline, glycine betaine), some occur rarely (such as trehalose, DMSP) whereas a few like ectoine are not known to be synthesized by the plants. Therefore, installing osmoprotectant synthesis has become a potential route for breeding stress-tolerant crops (Rathinasabapathi 2000). Accordingly, many genes involved in the synthesis and accumulation of these osmoprotectants have been explored for their potential in engineering plant abiotic-stress tolerance (Bhatnagar-Mathur et al. 2008; Vincour and Altman 2005). Several attempts have been made in recent years either to increase the biosynthesis or decrease the catabolism of these compounds in plants by transferring genes associated with their metabolism for developing abiotic stress-tolerant transgenics. The following sections focus on the current understanding of roles various osmolytes such as proline, ectoine, glycine betaine, sugars (trehalose) and sugar alcohols (mannitol, sorbitol, pinitol) play in imparting tolerance to plants against abiotic stresses along with the recent advances made in producing transgenic plants produced by using their biosynthetic pathway genes.

10.4.1 Proline

Proline has a distinct place among the osmolytes for the plethora of roles it plays in mitigating the deleterious effects of environmental stresses in several plant species. Its high solubility (1.54 kg L^{-1} water at 20 °C) Hyung et al. (2008) makes it an ideal solute. Proline is a proteinogenic amino acid with an exceptional conformational rigidity and is essential for primary metabolism too (Suprasanna et al. 2014). It acts as an osmoprotectant, and plays an important role in osmotic balancing, protection of sub-cellular structures, enzymes and in increasing cellular osmolarity that provide the necessary turgor for cell expansion (Kumar et al. 2010; Matysik et al. 2002; Sairam and Tyagi 2004; Yokoi et al. 2002). Proline has been shown to scavenge singlet oxygen and free radicals including hydroxyl ions and hence stabilize proteins, DNA as well as membranes (Alia et al. 2001; Kavi Kishor and Sreenivasulu 2014; Matysik et al. 2002). As a molecular chaperone, proline has also been demonstrated to protect the protein integrity and to increase the enzyme activities (Suprasanna et al. 2014). In addition, proline also acts as a source of carbon, nitrogen and energy during recovery from stresses (Kavi Kishor et al. 2005).

Since the first report on accumulation of proline in wilting rye grass (Kemble and MacPherson 1954), numerous studies have shown enhanced proline content in higher plants under different abiotic stresses (Szabados and Savoure 2010). Its accumulation has been reported in several glycophyte plants during conditions of drought (Choudhary et al. 2005), salinity (Kumar et al. 2008), high light and UV irradiation (Salama et al. 2011; Saradhi et al. 1995), heavy metals exposure (Radic et al. 2010; Sharma and Dietz 2006; Yilmaz and Parlak 2011), oxidative stress

(Thounaojam et al. 2012; Yang et al. 2009a) and in response to biotic stresses (Fabro et al. 2004; Haudecoeur et al. 2009). Similarly, proline accumulation is also a common adaptive response in halophytes to various abiotic stresses (Deuschle et al. 2001).

In higher plants, proline is synthesized by two pathways, first via arginine/ornithine and second via glutamate. Radioisotope labelling reveals that proline gets synthesized mainly via glutamate pathway under stress conditions. In this pathway glutamate is catalyzed to glutamatic-γ-glutamyl kinase (GSA) by pyrroline 5-carboxylate synthetase (P5CS) in plants and other eukaryotes. Pyrroline 5-carboxylate (P5C) is then reduced to proline by P5C reductase (P5CR) (Kavi Kishor et al. 2005). Proline catabolism occurs in mitochondria via the sequential action of proline dehydrogenase (PDH) or proline oxidase (POX), thus producing P5C from proline, and P5C dehydrogenase (P5CDH), which converts P5C to glutamate. PDH is encoded by two genes, whereas a single P5CDH gene has been identified in *Arabidopsis* and tobacco (Deuschle et al. 2001; Ribarits et al. 2007). An alternative precursor for proline biosynthesis is ornithine, which is transaminated first by ornithine-delta-aminotransferase (OAT), a mitochondrial-located enzyme producing GSA and P5C, which is then converted to proline (Delauney et al. 1993; Roosens et al. 1998; Verbruggen and Hermans 2008).

Almost all the genes involved in proline biosynthesis have been transferred to various economically-important crop plants for enhanced proline accumulation and consequent tolerance against diverse environmental stresses; pleotropic effects have been observed upon induction of constitutive expression of such genes (Kavi Kishor et al. 2005). An updated summarized list of various transgenic plants developed by transferring genes that govern the pathways involved in proline synthesis and degradation is given in Table 10.1. Figure 10.1 depicts the production of transgenic rice plants transformed with a mutagenized proline biosynthetic pathway gene P5CSF129A for enhanced proline accumulation and resultant salinity-stress tolerance (Kumar et al. 2010).

10.4.2 Glycine Betaine

Glycine betaine, an N-trimethyl derivative of glycine and a quaternary ammonium compound is one of the best-studied and most efficient compatible solutes (Chen and Murata 2011). It is a small organic metabolite, highly soluble in water but also contains non-polar moiety constituting 3-methyl groups. It is a non-toxic cellular osmolyte which raises the osmolarity of the cell during stress and recovery phases and thus plays an important role in stress mitigation (Gupta and Huang 2014; Wani et al. 2013). Due to its unique structural features, it interacts both with hydrophobic and hydrophilic domains of macromolecules including enzymes and proteins (Gupta and Huang 2014). Two biosynthetic pathways lead to the generation of glycine betaine (Chen and Murata 2002). In higher plants, it is biosynthesized from choline by a two-step oxidation reaction. The first oxidation is catalyzed by choline

Table 10.1 List of transgenics developed using proline biosynthetic pathway genes that conferred abiotic stress tolerance

Gene	Gene origin	Target plant	Enhanced tolerance and phenotype of transgenic plants	Reference
P5CS	*Vigna aconitifolia*	*Nicotiana tabacum*	Increased biomass production and enhanced flower and seed development under salt stress	Kavi Kishor et al. (1995)
P5CS	*V. aconitifolia*	*Oryza sativa*	Rice seedlings showed significantly higher tolerance to drought and salt stress	Su and Wu (2004)
P5CS	*V. aconitifolia*	*Larix leptoeuropaea*	Enhanced tolerance to cold, salinity and freezing stresses	Gleeson et al. (2005)
P5CS	*V. aconitifolia*	*Medicago*	Proline essential for the maintenance of nitrogen-fixing activity under osmotic stress	Verdoy et al. (2006)
P5CS	*Arabidopsis thaliana*	*N. tabacum*	Increased proline and osmotic stress tolerance	Yamchi et al. (2007)
P5CS	*V. aconitifolia*	*N. tabacum*	Proline accumulation and increased enzyme activities under salinity-induced oxidative stress	Razavizadeh and Ehsanpour (2009)
P5CS	*V. aconitifolia*	*Cicer arietinum*	Enhanced proline and salt tolerance	Ghanti et al. (2011)
P5CS	*A. thaliana*	*N. tabacum*	Increased proline content and salt tolerance	Jazii et al. (2011)
P5CS	*V. aconitifolia*	*O. sativa*	Better salt stress tolerance at 200 mM NaCl	Karthikeyan et al. (2011)
P5CS	Not mentioned	*Olea europaea*	Enhanced tolerance to salinity	Behelgardy et al. (2012)
P5CS1	*Phaseolus vulgaris*	*A. thaliana*	Increased plant tolerance to salt and drought stresses	Chen et al. (2010)
P5CS1	*P. vulgaris*	*A. thaliana*	Increased tolerance to salt stress	Chen et al. (2013)
P5CS2	*P. vulgaris*	*N. tabacum*	Proline accumulation and improved drought tolerance	Chen et al. (2008)
P5CSF129A (mutated)	*A. thaliana*	*N. tabacum*	Higher proline content and enhanced resistance to osmotic stress	Hong et al. (2000)
P5CSF129A	*V. aconitifolia*	*Carrizo citrange*	Drought tolerance and increased antioxidative capacity attributed to increased proline content	Molinari et al. (2004)
P5CSF129A	*V. aconitifolia*	*Cicer arietinum*	Enhanced proline and drought stress tolerance	Bhatnagar-Mathur et al. (2009)

(continued)

Table 10.1 (continued)

Gene	Gene origin	Target plant	Enhanced tolerance and phenotype of transgenic plants	Reference
P5CSF129A	*V. aconitifolia*	*O. sativa*	Enhanced proline, lower lipid peroxidation and better growth performance under salt stress	Kumar et al. (2010)
P5CSF129A	*V. aconitifolia*	*Swingle citrumelo*	Enhanced drought tolerance and antioxidant enzymatic activities	de Campos et al. (2011)
P5CSF129A	*V. aconitifolia*	*N. tabacum*	Mild but distinct positive effect on abiotic stress	Cvikrova et al. (2012)
P5CSF129A	*V. aconitifolia*	*Cajanus cajan*	Enhanced proline accumulation and salt tolerance	Surekha et al. (2014)
P5CR	*A. thaliana*	*Glycine max*	Better antioxidants levels under drought and heat stress simultaneously	Kocsy et al. (2005)
P5CR	*Triticum aestivum*	*A. thaliana*	Enhanced root growth under salt stress and decreased lipid peroxidation under salt, draught and ABA stress	Ma et al. (2008)
ProDH	*A. thaliana*	*N. tabacum*	Antisense plants showed increased proline content	Kochetov et al. (2004)
ProDH	*A. thaliana*	*N. tabacum*	Antisense suppression resulted in heavy metal tolerance	Kolodyazhnaya et al. (2007)
ProDH	*Brassica oleraceae italica*	*B. oleraceae*	Antisense ProDH resulted in low proline level	Yang et al. (2010)
ProDH	*A. thaliana*	*N. tabacum*	Antisense suppression showed elevated proline level and tolerance to various abiotic stresses	Ibragimova et al. (2012)
OAT	*A. thaliana*	*N. plumbaginifolia*	Overexpression increased proline levels and osmotic tolerance	Roosens et al. (2002)
OAT	*A. thaliana*	*O. sativa*	Increased proline content and improved yield under salt and drought stress	Wu et al. (2003)
OAT	*O. sativa*	*O sativa*	Drought and oxidative stress tolerance	You et al. (2012)

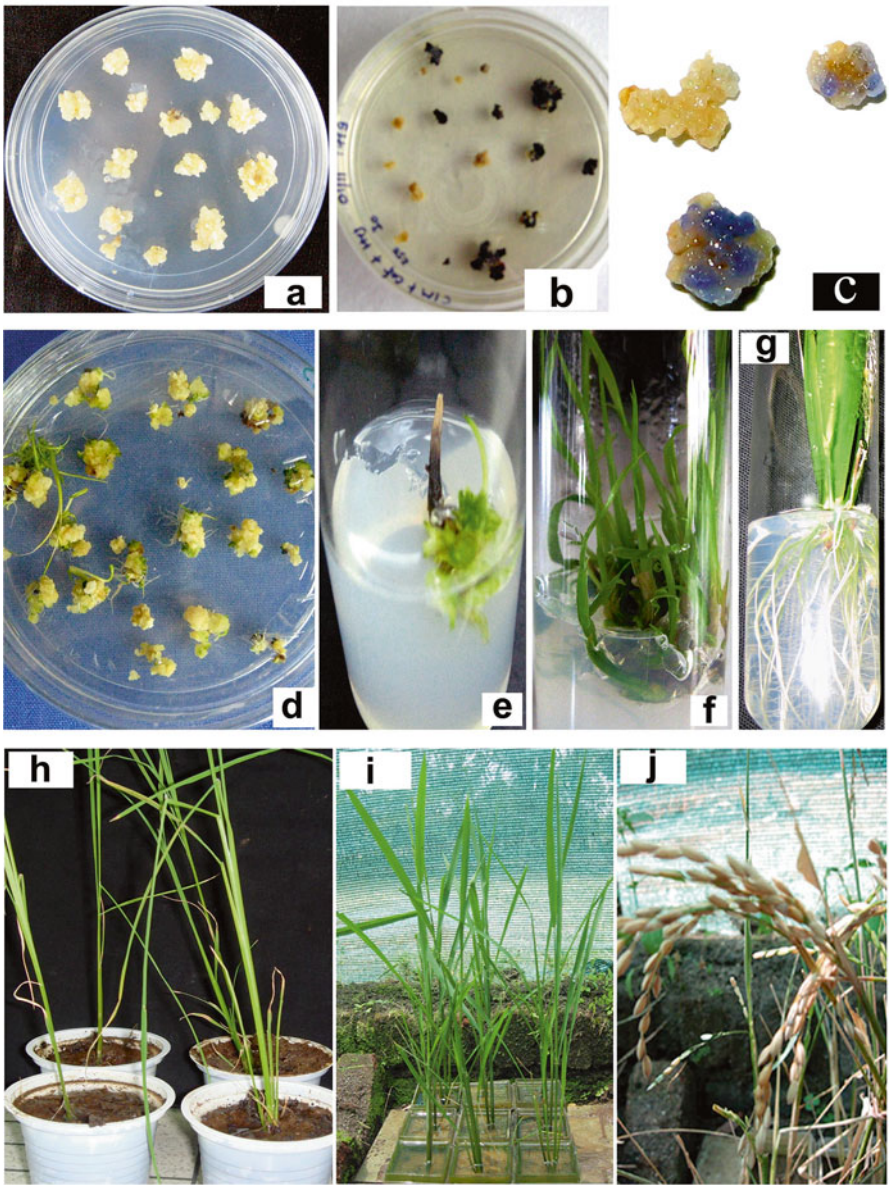

Fig. 10.1 Different stages of *Agrobacterium*-mediated transformation of *indica* rice cv. KJT-3 for over-expressing a mutagenized proline biosynthetic pathway gene P5CSF129A using mature embryo derived callus explants. **a** embryogenic-like callus placed on co-cultivation media; **b** selection of calli after co-cultivation on CIM containing 20 mg l^{-1} hygromycin and 250 mg l^{-1} cefotaxime; **c** GUS expression in callus tissues (*indigenous blue color*) after first cycle of antibiotic selection; **d** indirect shoot regeneration from antibiotic resistant putative transformant calli on regeneration media in presence of 20 mg l^{-1} hygromycin and 250 mg l^{-1} cefotaxime (albino plants can be seen); **e** Shoot initiation from shoot apex on antibiotic selection medium; **f** multiple shoot regeneration on antibiotic selection medium containing 30 mg l^{-1} hygromycin and 250 mg l^{-1} cefotaxime; **g** rooting of putative transformed shootlet on ½ MS; **h** acclimatized putative transformants growing in plastic cups in tissue culture laboratory; **i** hardened putative transformed plants of T_0 growing in the greenhouse at the Botanical Garden, Department of Botany, University of Pune; **j** primary transformants (T_0) at the grain-filling stage in the greenhouse (Source: Kumar et al. (2010) with permission of Springer Science + Business Media)

monooxygenase (CMO) and the second is catalyzed by NAD^+-dependent betaine aldehyde dehydrogenase (BADH) (Chen and Murata 2002; Takabe et al. 2006).

The biological functions of glycine betaine have been studied extensively in higher plants including spinach, sugar beet, barley and maize (Chen and Murata 2008). Glycine betaine is reported to be accumulated intracellularly at high concentration through synthesis, uptake or both and this amassing increases further under various abiotic stresses in a number of plants (Bhuiyan et al. 2007; Hassine et al. 2008; Hattori et al. 2009; Wang et al. 2010a). Glycine betaine has been reported to protect plants from antagonistic effects of a range of abiotic stresses by maintaining the water balance between plant cells and environment, osmotic adjustment, protecting the thylakoid membrane, protein stabilization and photosynthetic machinery protection (Chen and Murata 2008; Khan et al. 2009). Additionally, its exogenous application has also been known to confer stress tolerance and increase growth and survival (Chen and Murata 2011).

Interestingly though, the distribution of glycine betaine among plants is sporadic, and a number of economically-important crop species including tomato, potato and various genotypes of rice are known as non-accumulators of glycine betaine, and therefore, these species are potential targets for engineering betaine synthesis (Khan et al. 2009). The cloning of various genes encoding enzymes that catalyze the biosynthesis of glycine betaine has been reported, and many lines of transgenic plants have been produced expressing the glycine betaine biosynthetic genes from plants as well as from bacteria (Chen and Murata 2011); a summarized list of such events has been given in Table 10.2.

10.4.3 Ectoine

Many halophilic microbes, such as α- and γ- Proteobacteria and Actinobacteridae synthesize and accumulate this very important osmolyte to confer resistance to salinity and temperature stresses. It is considered as a heterocyclic amino acid or a partially-hydrogenated pyrimidine derivative (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) (Galinski et al. 1985) and is synthesized via three successive enzymatic reactions, starting from L-aspartate β-semialdehyde and requires three enzymes L-diaminobutyric acid acetyl transferase, L-diaminobutyric acid transaminase and L-ectoine synthase encoded by EctA, EctB and EctC genes, respectively (Moghaieb et al. 2011; Pastor et al. 2010). Nakayama et al. (2000) transformed tobacco cells with the genes codifying the ectoine biosynthesis from *Halomonas elongata* and the resultant transgenic tobacco plants showed ectoine accumulation correlated with tolerance to hyperosmotic stress. Ectoine was attributed to protection of stomatal conductance and carboxylation activity, thereby avoiding the reduced photosynthetic rate under salinity stress (Moghaieb et al. 2006). The enhanced salt tolerance of transgenic tobacco plants carrying the ectoine gene was credited to their better abilities for uptake of water through roots and its transport to shoots, besides enhanced nitrogen supply to leaves by increasing the

Table 10.2 List of transgenic plants that over-expressed glycine betaine biosynthetic pathway genes of bacterial and plant origin and their enhanced abiotic stress tolerance

Gene	Gene origin	Target plant	Enhanced tolerance and phenotype of transgenics	Reference
Bacterial origin genes for biosynthesis and accumulation of glycine betaine				
codA	*Arthrobacter globiformis*	*Arabidopsis thaliana*	Chilling and salt tolerance	Hayashi et al. (1998)
codA	*A. globiformis*	*A. thaliana*	Freezing tolerance with better photosynthetic potential	Sakamoto and Murata (2000)
codA	*A. globiformis*	*Brassica juncea*	Salt tolerance	Prasad et al. (2000)
codA	*A. globiformis*	*A. thaliana*	Salt tolerance	Sulpice et al. (2003)
codA	*A. globiformis*	*Oryza sativa*	Freezing tolerance	Konstantinova et al. (2002)
codA	*A. globiformis*	*Lycopersicon esculentum*	Chilling, salt and oxidative stress	Park et al. (2007)
codA	Not available	*Solanum tuberosum*	Salt, drought, oxidative stress	Ahmad et al. (2008)
cox	*Arthrobacter pascens*	*A. thaliana*	Salt, drought and freezing	Huang et al. (2000)
cox	*A. pascens*	*O. sativa*	Salt tolerance	Su et al. (2006)
betA	*Escherichia coli*	*Zea mays*	Drought tolerance	Quan et al. (2004)
BADH	*E. coli*	*Medicago sativa*	Salt tolerance	Liu et al. (2011a, b)
BADH	*E. coli*	*M. sativa*	Salt tolerance	Yan et al. (2012)
GSMT *and* DMT[a]	*Aphanothece halophytica*	*O. sativa*	Salt and cold stress tolerance	Niu et al. (2014)
Plant origin genes for biosynthesis and accumulation of glycine betaine				
BADH	*Hordeum vulgare*	*O. sativa*	Salt, cold and heat tolerance	Kishitani et al. (2000)
BADH	*Spinacia oleracea*	*Nicotiana tabacum*	Heat tolerance	Yang et al. (2007)
CMO + BADH	*S. oleracea*	*A. thaliana*	Salt tolerance	Hibino et al. (2002)
BADH	*Atriplex hortensis*	*Lycopersicon esculentum*	Salt tolerance	Zhou et al. (2007)
BADH1	*O. sativa*	*N. tabacum*	Salinity stress tolerance	Hasthanasombut et al. (2010)
BADH	*S. oleracea*	*Solanum tuberosum*	Drought and salinity tolerance	Zhang et al. (2011a, b)
CMO	*S. oleracea*	*O. sativa*	Salt and temperature	Shirasawa et al. (2006)
CMO	*Beta vulgaris*	*N. tabacum*	Salt and drought	Zhang et al. (2008)
BADH	*A. hortensis*	*Triticum aestivum*	Drought and heat	Wang et al. (2010a, b)
BADH	*Suaeda liaotungensis*	*Solanum lycopersicum*	Salt tolerance	Wang et al. (2013)
BADH	*S. oleracea*	*Lycopersicon esculentum*	Heat and photoinhibition tolerance	Li et al. (2014)

[a]Co-expression

GSMT glycine sarcosine methyltransferase, *SDMT* sarcosine dimethylglycine methyltransferase

transpiration and protecting the RuBisCo proteins from the deleterious effects of salinity, thereby improving the rate of photosynthesis (Moghaieb et al. 2006). Rai et al. (2006) produced transgenic tobacco plants via *Agrobacterium*-mediated genetic transformation using EctABC operon genes from *Miktoniscus halophilus* and the transcripts were targeted to chloroplast. The resultant transgenics showed enhanced salt tolerance 100–300 mM NaCl and their tolerance nature was correlated with the ectoine-induced higher stability of RuBisCo in chloroplasts. More recently, Moghaieb et al. (2011) transferred three *H. elongata* ectoine biosynthetic pathway genes (EctA, EctB, EctC) to tomato plants. Roots of transgenic tomato plants (T_3) showed ectoine accumulation which was further enhanced with increasing salinity. The transgenic lines exhibited better salt tolerance than their non-transformant counterparts with higher peroxidase activity and photosynthetic rate coupled with lower lipid peroxidation.

10.4.4 Sugars and Sugar Alcohols (Polyols)

Abiotic stresses severely affect the overall carbon metabolism and the levels of specific sugars (and polyols). Polyols are the compounds with multiple hydroxyl functional groups for organic reactions. These sugars, such as fructose, trehalose and a few sugar alcohols (a class of polyols) such as mannitol and sorbitol are known to accumulate under abiotic stresses and contribute significantly towards stress alleviation via osmotic adjustment, carbon storage, ROS scavenging and overall osmotic adjustment, besides their roles as low molecular weight chaperons (Gupta and Huang 2014; Parvaiz and Satywati 2008). A decrease in starch content and an increase in both reducing and non-reducing sugars and polyphenol levels are reported in leaves of *Bruguiera parviflora* (Parida et al. 2002).

Among various sugars, trehalose is perhaps the most important and sought after compound for its diverse physiological roles under abiotic stresses. Trehalose is a rare and non-reducing disaccharide, present in many bacteria and fungi besides some desiccation-tolerant higher plants, also known as resurrection plants as they tolerate complete dehydration and have the ability to spring back to life upon rehydration. The fundamental role of trehalose includes protection of cellular membranes and proteins from stress-induced damage (Garg et al. 2002). It plays a crucial role in metabolic homeostasis and signal transduction pathways involved in cell proliferation and differentiation (Turan et al. 2012). Trehalose also increases the thermostability and thermoactivities of reverse transcriptase and proteins. Trehalose gets synthesized in two steps from glucose-6-phosphate and uridine diphosphoglucose, via trehalose-6-phosphate. The first step is mediated by trehalose phosphate synthase (TPS), and the second by trehalose-6-phosphate phosphatase (TPP) although non-specific phosphatases can also carry out this reaction. The genes involved in the regulation of its metabolism are seen as

important targets for genetic engineering for its enhanced synthesis and accumulation for stress mitigation.

Several researchers have used trehalose biosynthetic pathway genes to target a number of plant species for enhanced abiotic stress tolerance. The initial attempts were made to produce transgenic tobacco plants expressing TPS genes independently from *Escherichia coli* or yeast and the resulting plants displayed improved drought tolerance (Holmstrom et al. 1996; Pilon-Smits et al. 1998). Transgenic tomato plants carrying the TPS gene have also been reported for their enhanced drought tolerance (Cortina and Culianez-Macia 2005). Garg et al. (2002) transferred *E. coli* trehalose biosynthetic genes otsA and otsB (TPS homologs) as a fusion gene to rice and the resultant transgenic plants accumulated 3–10 times higher trehalose than their non-transformed counterparts. The transgenic lines exhibited sustained plant growth, less photo-oxidative damage, and more favorable mineral balance under salt, drought and low-temperature stress conditions. Jang et al. (2003) expressed bifunctional fusion of *E. coli* genes for TPS and TPP in transgenic rice plants, which provided abiotic stress tolerance to the transgenic plants. Similarly, in tobacco, heterologous expression of AtTPS1 gene from *Arabidopsis* increased tolerance to several abiotic stresses such as drought, desiccation and temperature stresses (Almeida et al. 2005). The modest increase in trehalose levels in the transgenic plants resulted in a higher photosynthetic rate and decrease in photo-oxidative damage during stress (Vincour and Altman 2005). The low levels of trehalose in transgenic plants may be due to specific trehalase activity, which degrades trehalose; hence, it might be possible to increase trehalose accumulation by down-regulating trehalase activity (Suprasanna 2003). However, even this modest increase in trehalose levels in transgenic plants resulted in significantly higher photosynthetic rate with lesser photo-oxidative damage during stress. Miranda et al. (2007) reported an interesting finding, as the authors constructed a bifunctional yeast TPS1-TPS2 fusion and in the resultant transgenic *Arabidopsis* plants, chimeric fusion improved extreme drought, heat, freezing and salt stress conditions and, unlike previous reports, without causing morphological abnormalities. More recently, Suarez et al. (2009) have also used the same chimeric gene construct under the control of rd29A promoter for producing transgenic alfalfa plants and the obtained plants displayed a significant increase in tolerance against multiple abiotic stresses.

Another important osmolyte from the groups of sugars and sugar alcohols is mannitol, a 6-carbon sugar alcohol, which accumulates upon and alleviates abiotic stresses (Vincour and Altman 2005). Its accumulation is regulated by the inhibition of competing pathways and decreased mannitol consumption. However, despite its wide distribution in nature, it has received relatively less attention from researchers. Nevertheless, various reports have clearly indicated that plant species that accumulate mannitol hold an advantage over others as it offers physiological roles to protect plants against stress conditions. Therefore, some researchers have attempted genetic engineering approaches for its enhanced accumulation in plants and to provide stress tolerance. The mannitol dehydrogenase

enzyme, encoded by the bacterial mtlD gene, is the key enzyme in mannitol metabolism, reversibly converting fructose-6-phosphate to mannitol-1-phosphate. The mtlD gene has been transferred to several crops for mannitol overaccumulation, often resulting in their better growth performances under salinity and/or drought conditions. Thomas et al. (1995) transferred bacterial mtlD gene into *Arabidopsis* and the transformed plants showed better seed germination and physiological characteristics up to 400 mM NaCl stress. Similarly, Abebe et al. (2003) demonstrated that the expression of the *Escherichia coli* mtlD gene in third generation transgenic wheat resulted in an improved tolerance to salinity and exhibited enhanced fresh weight, dry weight and plant height as compared with the wild-type non-transgenic wheat plants. In another instance, Rahnama et al. (2011) introduced the bacterial mtlD gene into potato by *Agrobacterium*-mediated transformation for enhancing the salt tolerance of transgenics. The authors attributed the improved tolerance of transgenic plant to the induction and progressive accumulation of mannitol in their roots and shoots. In an interesting study, Nguyen et al. (2013) successfully attempted a transgene pyramiding of *Hordeum vulgare* HVA1 and bacterial mannitol-1-phosphate dehydrogenase (mtlD) under the regulation of rice actine Act1; a combination of these genes was transferred into the maize genome and the genes conferred tolerance to salinity and drought.

In addition to trehalose and mannitol, other group members with noticeable osmoprotectant properties such as inositol, sorbitol and pinitol have also been engineered for their enhanced biosynthesis and tolerance. Japanese persimmon was transformed with apple cDNA for S6PDH encoding NADP dependent sorbitol-6-phosphate dehydrogenase, accumulated sorbitol and showed higher salinity tolerance than untransformed plants as reflected by a higher ratio of variable to maximum fluorescence (Fv/Fm) Gao et al. (2001). Likewise, improved salt and drought tolerance was observed in *Nicotiana tabacum*, when transformed with cDNA of imt1 encoding for myo-inositol-o-methyltransferase, which was attributed to the accumulation of methylated inositol D-ononitol and higher CO2 fixation capacity in transgenic plants under stress condition (Sheveleva et al. 1997). In another investigation, the introgression and functional expression of either the PcINO1 (myo-inositol 1-phosphate synthase coding gene from the wild halophytic rice *Porteresia coarctata*) or McIMTI (inositol methyl transferase coding gene from common ice plant *Mesembryanthemum crystallinum*) has been shown to confer salt tolerance to transgenic tobacco plants (Majee et al. 2004; Sheveleva et al. 1997). More recently, Patra et al. (2010) co-expressed these two genes in cytosol and chloroplasts and the resultant tobacco plants accumulated a higher amount of inositol (free as well as methyl inositol). A positive correlation between the elevated level of total inositol and/or methylated inositol and the capability of the double transgenic plants to withstand a higher degree of salt stress compared to the plants expressing either PcINO1 or McIMT1 alone was inferred.

10.5 Reactive Oxygen Species (ROS) Formation Under Abiotic Stress

An unfortunate consequence and one of the most important and best documented attributes of abiotic stresses is the over-accumulation of ROS like $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ in plant cells (Hossain et al. 2014; Mittler et al. 2004). ROS are normally produced in different cellular compartments during normal physiological processes like photosynthesis and respiration; however, their rate of production is sharply increased under abiotic and biotic stress resulting in oxidative stress (Hossain and Fujita 2013; Hossain et al. 2010, 2013a, b, 2014; Kotchoni et al. 2006; Mittler 2002). Elevated levels of ROS lead to the inactivation of proteins and inhibit the activity of multiple enzymes, and result in the oxidation of other macromolecules and finally death of the plant. Therefore, ROS levels must be carefully controlled by maintaining adequate levels of antioxidants to perform their signaling functions (Miller et al. 2008; Mittler 2002; Petrov and Van Breusegem 2012).

The chloroplast is the vital source of ROS resulting in its generation in several locations, such as the electron transport chain (ETC), photosystem (PS I) and photosystem (PS II). A reduced rate of photosynthetic carbon fixation has been evidenced under various abiotic stresses including salinity, drought, temperature and heavy metals (Abogadallah 2010; Cruz de Carvalho 2008; Kaushal et al. 2011; Kim and Portis 2004; Sanda et al. 2011; Wise 1995).

In response to Cd stress, the essential metal ions (Ca^{2+} and Mn^{2+}) present in the PS II reaction center can be replaced, thereby limiting the photo system reaction and leading to uncoupling of electron transport in chloroplast (Atal et al. 1991; Baszynski et al. 1980; Mohanty and Mohanty 1988). Additionally, substitution of Mg^{2+} ions by Cd^{2+} ions can alter ribulose 1,5-bisphosphate carboxylase (RuBisCO) to favor the oxygenation reactions (Hossain et al. 2014; Krantev et al. 2008; Siedlecka and Baszynaski 1993; Siedlecka et al. 1998). The stimulation of the oxygenase activity of RuBisCo and restricted CO_2 fixation enhances the photorespiration resulting in H_2O_2 production in peroxisomes (Jaspers and Kangasjarvi 2010; Szarka et al. 2012).

Plant mitochondria can produce ROS at several sites along the ETC during the normal respiration process. Most ROS formation in mitochondria mainly occurs in the zone of complex I and III where $O_2^{\bullet-}$ is generated and rapidly converted into H_2O_2 (Bose et al. 2013). Up-regulation of ROS production as a result of ETC perturbations has been reported in plants exposed to salt stress (Hernandez et al. 1993; Mittova et al. 2003), chilling (Prasad et al. 1994a, b), high temperature (Schwarzlander et al. 2009) and Cd stress (Bi et al. 2009; Schwarzlander et al. 2009). Although, mitochondrial ROS production is much lower when compared to chloroplast ROS production, mitochondrial ROS are important regulators of a number of cellular processes, including stress adaptations and programmed cell death (Hossain et al. 2014; Robson and Vanlerberghe 2002).

Plant peroxisome is also another major site of H_2O_2 production during photorespiration, β-oxidation of fatty acids, by the enzymatic reactions of flavin oxidases as well as by the disproportionation of $O_2^{\bullet-}$. Abiotic stresses that hamper the CO_2 fixation in chloroplasts, cause glycolate to move to peroxisomes, where it is oxidized by glycolate oxidase (GO) forming H_2O_2 (Gechev et al. 2006; Takahashi and Murata 2008). Xanthine oxidase (XO) couple to SOD is also responsible for the production of H_2O_2 from O_2 in peroxisomes (Mhamdi et al. 2010). Apoplastic enzymes, such as cell-wall peroxidises and NADPH oxidases, are the main sources of $O_2^{\bullet-}$ and H_2O_2 and have been found to be stimulated in response to salt and heavy-metal stress (Abogadallah 2010; Remans et al. 2010; Wen et al. 2012). $O_2^{\bullet-}$ and H_2O_2 are also produced by other oxidases induced by abiotic and biotic stresses (Dat et al. 2000).

10.5.1 ROS Scavenging and Detoxification in Plant Cells

To overcome and withstand oxidative stress, plant cells are armed with sophisticated antioxidative systems including both enzymatic and non-enzymatic compounds that modulate cellular ROS concentration and setting cellular redox homeostasis. Enzymatic antioxidant defense in plants comprises mainly superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), catalase (CAT), glutathione peroxidase (GPX), glutathione *S*-transferase (GST) and peroxidase (POX); whereas, non-enzymatic antioxidant defense includes ascorbate (AsA), glutathione (GSH), tocopherol, carotenoids, flavonoids and proline in different sub-cellular organelles (Apel and Hirt 2004; Hossain and Fujita 2013). The delicate balance between ROS production and scavenging that allows this duality in function to exist in plants is thought to be orchestrated by a large network of genes that tightly regulate ROS production and scavenging (Miller et al. 2008; Petrov and Van Breusegem 2012). Recent studies in plants have demonstrated that appropriate levels of ROS (especially H_2O_2) activates multiple defense responses that reinforce resistant to various environmental stresses (Petrov and Van Breusegem 2012).

10.5.2 Genetic Engineering for Abiotic Oxidative Stress Tolerance

Attempts to reduce oxidative damage under stress conditions have included the manipulation of ROS scavenging enzymes by gene transfer technology. A number of transgenic plants overexpressing genes encoding ROS detoxifying enzymes or non-enzymatic antioxidants have been reported for enhanced abiotic oxidative stress tolerance (Table 10.3).

Table 10.3 Transgenic plants for ROS scavenging enzymatic and non-enzymatic antioxidant genes and their roles for abiotic stress tolerance

Gene	Host plant	Findings/proposed mechanisms/reported phenotype	Reference
Cu/Zn SOD	*Oryza sativa*	Transgenic plants showed better salinity and drought tolerance and methyl viologen (MV)-induced oxidative stress tolerance	Prashanth et al. (2008)
MnSOD	*Arabidopsis thaliana*	Genetically engineered plants showed better drought tolerance due to improved ROS detoxification	Liu et al. (2013a)
MnSOD	*Lycopersicon esculentum*	Transgenic tomato plants showed better growth, higher SOD and APX activity under salt and MV induced oxidative stress	Wang et al. (2007a, b)
MnSOD	*Gossypium hirsutum*	Transgenic plants acquired improved drought tolerance through enhanced development of root and leaf and ROS scavenging	Zhang et al. (2014)
APX	*A. thaliana*	Transgenic plants showed salt tolerance due to higher APX activity, lower ROS accumulation and lipid peroxidation	Wei-Feng et al. (2008)
APX	*N. tabacum*	APX overexpressed plants showed enhanced salt, water deficit, MV, paraquat and PEG induced oxidative stress tolerance and increased net photosynthesis	Badawi et al. (2004)
APX	*N. tabacum*	Transgenic plants showed higher salinity and osmotic stress tolerance as indicated by higher APX activity and lower accumulation of H_2O_2 and higher photosynthetic rates	Sun et al. 2010b
APX	*N. tabacum*	Transgenic plants showed salt and drought stress tolerance, maintained higher plant growth under stress conditions	Singh et al. (2013)
APX	*O. sativa*	Transformed plants showed higher APX activity, better ROS detoxification and spikelet fertility under cold stress	Sato et al. (2011)
MDHAR	*N. tabacum*	MDHAR over-expressed plants exhibited higher MDHAR activity and AsA content and photosynthetic function	Eltayeb et al. (2007)
MDHAR	*N. tabacum*	Overexpressed plants have enhanced APX and MDHAR activities and have reduced levels of lipid peroxidation	Kavitha et al. (2010)
MDHAR	*L. esculentum*	Transgenic plants have higher MDHAR and APX activities, higher photosynthesis and oxidative stresses tolerance	Li et al. (2010)
MDHAR	*O. sativa*	Transgenic plants have higher MDHAR activity under salt stress and showed better plant growth and yield	Sultana et al. (2012)
DHAR	*N. tabacum*	Transgenic plants showed enhanced tolerance to drought, O_3, salt, and PEG induced oxidative stresses and showed higher net photosynthesis and better ascorbate redox state	Eltayeb et al. (2006)

(continued)

Table 10.3 (continued)

Gene	Host plant	Findings/proposed mechanisms/reported phenotype	Reference
DAHR	*Solanum tuberosum*	Transgenic plants exhibited faster growth under salt and drought stress, maintained higher DHAR activity and AsA	Eltayb et al. (2011)
DAHR	*L. esculentum*	Transgenic plants showed better seedling growth and higher salinity and MV-induced oxidative stress tolerance	Li et al. (2012)
GR	*O. sativa*	Genetically engineered plants showed improved protection against photo-bleaching of chlorophyll and photo-oxidative action of MV in thylakoid membranes at 25 °C	Kouril et al. (2003)
GPX	*N. tabacum*	Transgenic showed oxidative stress tolerance due to higher ROS detoxification induced by salt, chilling, light and MV	Yoshimura et al. (2004)
GPX	*A. thaliana*	Transgenic plants were more tolerant to oxidative stress caused by chilling, salinity, drought, H_2O_2, and MV	Gaber et al. (2006)
GST	*A. thaliana*	Transgenic plants showed enhanced salt tolerance as indicated by lower MDA and higher seedling germination	Qi et al. (2010)
GST	*N. tabacum*	Transgenic plants showed higher salinity and dehydration tolerance by maintaining better root and shoot growth	Ji et al. (2010)
GST	*N. tabacum*	Transgenic plants showed higher seed germination, survival and better growth compared to wild type under salt stress	Jha et al. (2011)
GST	*N. tabacum*	GST overexpressed plants showed normal growth under drought, NaCl, and Cd stresses and maintained higher GST activity and a significantly lower level of ROS	Liu et al. (2013b)
CAT	*Corchorus olitorius*	Transgenic plants showed better tolerance to salt stress and exhibited normal growth characteristics	Islam et al. (2013)
CAT	*O. sativa*	Transgenic plants showed better cold tolerance due to lower ROS accumulation and higher CAT activity	Matsumura et al. (2002)
γ-ECS	*O. sativa*	Transgenic plants showed better tolerance and germination rate under abiotic stress including salinity by improving redox homeostasis via an enhanced GSH pool	Choe et al. (2013)
γ-ECS	*O. sativa*	Genetically engineered plants showed higher salinity tolerance by modulating redox homeostasis, lower ion leakage in the presence of MV and salt	Bae et al. (2013)
GalUR	*S. tuberosum*	Transgenic showed higher APX, DHAR, GST, GPX, GR, Gly I and Gly II activities and maintain higher AsA and GSH level and their redox state and restricted the increase of MG	Upadhyaya et al. (2011)
GLOase	*S. tuberosum*	Transgenic plants showed higher AsA level and tolerance to abiotic stresses induced by MV, NaCl or mannitol	Hemavathi et al. (2010)

(continued)

Table 10.3 (continued)

Gene	Host plant	Findings/proposed mechanisms/reported phenotype	Reference
GalUR	*S. tuberosum*	Transgenic plans showed nearly twofold increase in AsA content enhanced tolerance to NaCl or mannitol	Hemavathi et al. (2009)
GalUR	*S. tuberosum*	Transgenic tubers under abiotic stress enhanced the activities of SOD, CAT, APX, DHAR, GR as well as the levels of AsA, GSH and proline which imparts improved tolerance	Hemavathi et al. (2011)
GLOase	*L. esculentum*	Higher endogenous AsA level up-regulate the antioxidant system which imparts improved tolerance against stresses	Lim et al. (2012)
GME	*L. esculentum*	The transgenic plants showed a higher survival and growth under salt stress. Improved tolerance was closely related to higher AsA content that increased ROS scavenging	Zhang et al. (2011b)
GalLDH	*N. tabacum*	Transgenic plants showed higher AsA level and higher resistance to oxidative stress caused by salt and paraquat	Liu et al. (2013c)
GalLDH	*A. thaliana*	The transgenic plants showed higher AsA levels and tolerance to a range of various abiotic stresses like salt, cold, and heat	Lisko et al. (2013)
GST + CAT	*O. sativa*	Co-expression of the GST and CAT1 resulted in the enhancement of the ROS-scavenging system that led to increased oxidative stress protection	Zhao and Zhang (2006)
CuZnSOD + APX	*Ipomoea batatas*	Transgenic plants showed higher salt tolerance due to the increased activities of SOD, APX, POD and CAT. The transgenic plants also showed lower oxidative and chlorophyll damage	Wang et al. (2011)
GST + CAT	*O. sativa*	Transgenic plants under Cd stress and combined stress (Cd and heat) conditions showed a sharp increase in CAT, GST, APX, MDHAR, DHAR, GR activities and higher oxidative stress tolerance	Zhao et al. (2009)
CuZnSOD + APX+ DHAR	*N. tabacum*	Tobacco plants overexpressing three antioxidant enzymes (CuZnSOD, APX and DHAR) showed greater tolerance to oxidative stress than double transgenic (CuZnSOD and APX) due to better ROS management	Lee et al. (2007, b)
Cyt SOD + Cyt APX	*N. tabacum*	Transgenic plants over-expressing both enzymes showed higher MDHAR, DHAR, GST, POX and CAT activities and better oxidative stress tolerance	Faize et al. (2011)
SOD+ APX + codA	*S. tuberosum*	Transgenic plants overexpressing SOD, APX and codA genes showed enhance protection to salt and drought stress as compare to double transgenic (SOD + APX)	Ahmad et al. (2010)

[a]*γ-ECS* gamma-glutamylcysteinyl, *GalUR* D-galacturonic acid reductase, *GLOase* L-gulono-gamma-lactone oxidase, *GalLDH* l-Galactono-1, 4-lactone dehydrogenase, *GME* GDP- Mannose 3′,5′-epimerase

Transgenic plants overexpressing different isoforms of SOD in different plant species induces oxidative stress tolerance and showed improved plant growth and development under salt and drought stress (Liu et al. 2013a; Prashanth et al. 2008; Wang et al. 2007a, b, 2010a, b; Zhang et al. 2014). Improved growth and development were also observed due to efficient ROS regulation in transgenic plants overexpressing ascorbate-glutathione cycle enzymes (APX, MDHAR, DHAR, GR) under various abiotic stress conditions (Badawi et al. 2004; Eltayeb et al. 2006, 2007, 2011; Kavitha et al. 2010; Kouril et al. 2003; Li et al. 2010, 2012; Singh et al. 2013; Sultana et al. 2012; Sun et al. 2010b; Wei-Feng et al. 2008). Likewise, improved oxidative tolerance in transgenic plants overexpressing CAT, GST and GPX genes has also been reported (Gaber et al. 2006; Islam et al. 2013; Jha et al. 2011; Ji et al. 2010; Liu et al. 2013b; Matsumura et al. 2002; Qi et al. 2010; Yoshimura et al. 2004).

Apart from enzymatic antioxidant genes, recently a large number of transgenic plants overexpressing non-enzymatic antioxidant genes (γ-ECS: gamma-glutamylcysteinyl; GalUR: D-galacturonic acid reductase; GLOase: L-gulono-gamma-lactone oxidase; GalLDH: l-Galactono-1, 4-lactone dehydrogenase; GME: GDP- Mannose 3′,5′-epimerase) responsible AsA and GSH biosynthesis have been reported for improved abiotic oxidative stress tolerance (Hemavathi et al. 2009, 2010, 2011; Lim et al. 2012; Lisko et al. 2013; Liu et al. 2013c; Upadhyaya et al. 2011; Zhang et al. 2011a). Importantly, some of the single gene transformants showed profound effects in ROS detoxification systems through the modulation of a number of antioxidant enzyme activities and multiple stress responsive pathways i.e. ROS and methylglyoxal (MG) detoxification pathways (Hemavathi et al. 2011; Upadhyaya et al. 2011; Wani and Gosal 2011); however, this phenomenon is found to be species specific in a majority of the cases. In an attempt to produce transgenic *indica* rice tolerant to salinity stress, Wani and Gosal (2011) reported in vitro screening of PCR-positive transgenic plantlets in which transgenic plants showed normal growth at 150 mM NaCl, whereas control plantlets turned yellow and ultimately did not survive (Fig. 10.2a–c).

Fig. 10.2 (continued) **c** Scuteller-derived embryogenic callus with seed (*A*); Target plate used for bombardment (*B*); Stereoscopic view of bombarded calli 48 h after bombardment (*C*); Selection of bombarded calli: non-transformed calli on medium without hygromycin (*left*), non-transformed calli on medium containing hygromycin (*middle*) and transformed calli on medium containing hygromycin (*right*) (*D*); Transient GUS expression after 1 week of selection (*E*); OsglyII transgenic calli showing increased fresh mass on medium containing 90 mM NaCl (*F*); Control calli showing necrosis and decrease in fresh mass on medium containing 90 mM NaCl (*G*); Shoot differentiation from bombarded calli (*H*); Rooting of putative transgenic plantlets (*I*); Stereoscopic view of T_0 rice leaf cutting showing GUS expression and control leaf (*J*); Stereoscopic view of T_0 rice root cuttings showing GUS expression and control root cuttings (*K*); Population of Osgly II putative transgenic plants in glasshouse (*L*); OsglyII transgenic and control plantlets after a shock of 150 mM NaCl for 15 days in MS liquid medium (*M*); PCR amplification of Osgly II in transgenic rice plants (*N*); Putative transgenic plants grown in the earthen pots showing normal flowering (*O*). The gene construct used in the study was kindly provided by Prof. Dr. S.K. Sopory, International Center for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India (Source: Wani and Gosal (2011) with permission of Springer Science + Business Media)

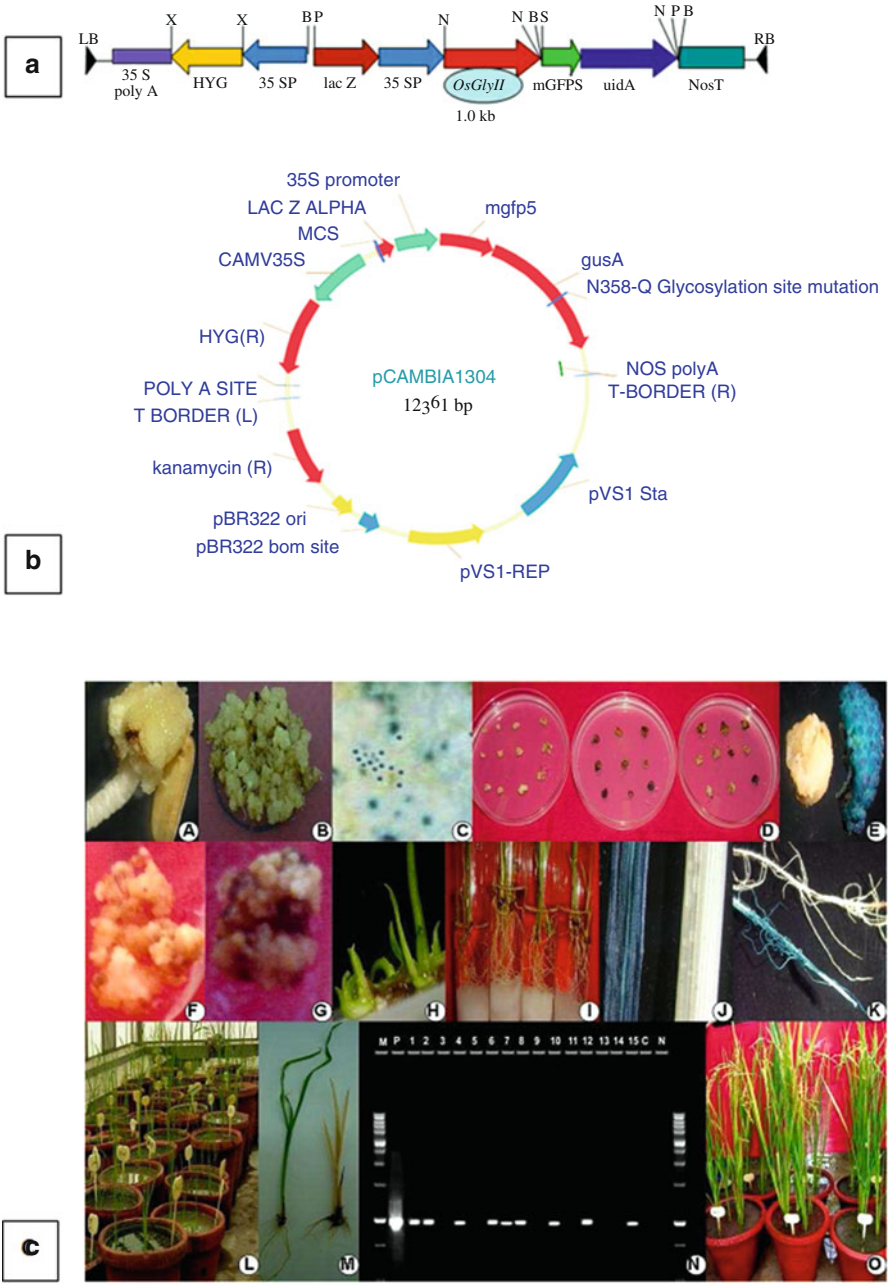

Fig. 10.2 **a** The transgene used in this study was glyoxalase II (osglyII) from *Oryza sativa* (GenBank accession no. AY054407) osglyII gene was cloned in pCAMBIA1304 plant transformation vector at NcoI and NcoI restriction sites. **b** This vector had nptII (neomycin phosphotransferase) and hptII (hygromycin phosphotransferase) genes as the selectable markers for bacteria and plants, respectively. It had both GUS and green fluorescent protein (mGFP) as the reporter genes.

10.5.3 Single Gene Transgenic Versus Multiple Genes

A wealth of examples of transgenic plants is available that show that overexpressing the genes encoding for antioxidants enhances tolerance to various abiotic oxidative stress; however, greater susceptibility or no improvements in transgenic plants have also been reported. These divergences have usually been attributed to the complexity of the ROS production and scavenging pathways, because modification of one enzyme or non-enzymatic antioxidants may not affect the pathways as a whole. Therefore, pyramiding of two or more genes has been found to be an alternative approach to increase the tolerance at a desired level (Ahmad et al. 2010; Lee et al. 2007b). Importantly, a number of transgenic plants showed that co-expression of multiple antioxidant genes in one genotype (Table 10.3) have better oxidative stress tolerance as compared to single gene transformants (Ahmad et al. 2010; Faize et al. 2011; Lee et al. 2007b; Wang et al. 2011; Zhao and Zhang 2006; Zhao et al. 2009). These results indicated that co-regulation among antioxidant enzymes is essential to maintain the correct balance between overproduction of ROS and their scavenging to keep them at the required levels to execute their signaling function and to improve oxidative stress tolerance.

10.5.4 Gene Pyramiding and Oxidative Stress Tolerance

Development of durable abiotic oxidative stress tolerant crop varieties may be achieved by pyramiding two or more genes through multiple transformation or crossbreeding. As for example, co-expression of APX and Cu/ZnSOD in transgenic plants of cassava, potato, tobacco, sweet potato, tall fescue and plum led to increased oxidative stress tolerance induced by salinity, chilling, drought, high temperate, herbicide and methyl viologen (Diaz-Vivancos 2013; Faize et al. 2011; Kwon et al. 2002; Lee et al. 2007b; Tang et al. 2006; Xu et al. 2014). Co-expression of the *Suaeda salsa* glutathione S-transferase (GST) and CAT1 in rice also gave tolerance to stresses caused by salt and paraquat (Zhao et al. 2006). Additionally, Ahmad et al. (2010) showed that transgenic potato plants overexpressing three genes (choline oxidase (codA), SOD, APX) provide enhanced abiotic stress tolerance as compared to single or double gene transformants. The above findings indicate that the combination of transgenes encoding different ROS-scavenging enzymes in various subcellular compartments might have a synergistic effect in improving stress tolerance. However, it is still necessary to expand our understanding on isolation of robust gene responsible for multiple stress tolerance and interaction of these genes in regulating multiple stress metabolic pathways. Likewise, refinement of the existing technique to be required for coordinated multigene manipulation in plants to provide more durable resistance against multiple abiotic stresses. Additionally, to generate oxidative stress tolerance it is of paramount importance to target the location, control the level and time of expression and ensure precursor availability for each enzyme in order to avoid negative effects (Kwon et al. 2001).

10.6 Transcription Factors (TFs)

Many genes are regulated for abiotic stresses like drought, salinity, heat, cold and mechanical wounding and their gene products help in providing stress tolerance to plants. These proteins can be categorized into two groups. The first group includes functional proteins, namely the late embryogenesis abundant (LEA) proteins; the second group is comprised of proteins that are regulatory in nature and further regulate signal transduction and stress-responsive gene expression. These include various transcription factors (TFs). TFs are of key importance in generating specificity in plant stress responses (Chen et al. 2002). Tolerance to abiotic stress may be achieved through the modification of endogenous plant pathways, often by manipulating important regulatory proteins such as transcription factors.

10.6.1 Role of Transcription Factors in Abiotic Stress

Being highly dynamic in nature, any biological system continuously changes responding to environmental and genetic perturbations. Plants are exposed to many types of environmental conditions during their life cycle; for example, soil moisture, salt concentration and temperature. Every year significant losses occur due to sudden frost and unusual freezing temperatures in winter and late cold spring (Heidarvand and Amiri 2010). More than 10 % of arable land is affected by drought and salinity, due to this there is more than a 50 % reduction in average yields of important crops worldwide (Bray et al. 2000). When a plant is subjected to abiotic stresses, an assortment of genes with diverse functions are induced or repressed. In stress condition, plants change at the whole-plant, tissue, cellular, physiological and molecular levels.

Transcription factors are master regulators that control gene clusters. A single TF can control the expression of many target genes through specific binding of TF to cis-acting elements in promoters of respective target genes. Plant genomes assign approximately 7 % of their coding sequences to TFs, which proves the complexity of transcriptional regulation (Udvardi et al. 2007). About 6 % of the genes in the rice and *Arabidopsis* genomes encode transcription factors, and nearly 45 % of them are plant specific (Kikuchi et al. 2003; Riechmann et al. 2000). It has been reported that overexpression of several genes encoding transcription factors resulted in stress tolerance of the plant. For example, overexpression of the ethylene response factor-like gene SUB1A in rice delayed leaf senescence and improved tolerance to submergence, drought and oxidative stress (Fukao et al. 2011). Rice plants overexpressing OsMYB55 showed an increase in total amino acid content and increased tolerance to high temperatures (El-Kereamy et al. 2012). In the *Arabidopsis thaliana* genome about 1500

TFs are described which are considered to be involved in stress responsive gene expression (Ratcliffe and Riechmann 2002; Riechmann et al. 2000). These TFs can be classified into several families based on the structure of their binding domains. Of a number of TFs listed elsewhere (Gosal et al. 2009), members of the MYB, MYC, ERF, bZIP and WRKY transcription factor families have already been implicated in the regulation of stress responses (Schwechheimer et al. 1998; Singh et al. 2002). Hence, bioengineering of the TFs of stress-signaling pathways to produce stress tolerant plants is one of the major goals of current biotechnology research.

Several pathways that independently respond to environmental stresses in both ABA dependent and independent ways, suggest that stress tolerance is controlled at the transcription level by an extremely intricate gene regulatory network (Bray 2004; Denby and Gehring 2005; Shinozaki et al. 2003; Umezawa et al. 2006). This type of transcriptional regulatory system is called a *regulon*. Several regulons involved in abiotic stress responses have been identified in *Arabidopsis* (Nakashima et al. 2009; Qin et al. 2011). The dehydration-responsive element binding protein 1 (DREB1)/C repeat binding factor (CBF) regulon functions in the cold-stress response, whereas the DREB2 regulon acts in heat and osmotic stress responses (Mizoi et al. 2011). The abscisic acid (ABA) responsive element (ABRE) binding protein (AREB)/ABRE binding factor (ABF) regulon functions in ABA-dependent gene expression under osmotic-stress conditions (Fujita et al. 2011). In addition, the NAC regulon is shown to be involved in osmotic-stress responses (Nakashima et al. 2009).

10.6.2 Some Successful Examples

Recently, Ravikumar et al. (2014) developed a transgenic rice using the AtDREB1A transcription factor. They developed transgenic rice plants with AtDREB1A in the background of *indica* rice cultivar Samba Mahsuri through *Agrobacterium*-mediated transformation. Expression of AtDREB1A was induced by drought stress in transgenic rice lines, which were highly tolerant to severe water-deficit stress in both the vegetative and reproductive stages without affecting their morphological or agronomic traits. Liu et al. (1998) reported upregulation of 12 stress-related genes in CaMV35S::AtDREB1A plants that showed twofold higher expression than in control plants. Six of these genes were known as stress-related, while the other six were found to have sequence similarities with cold acclimatization proteins. Overexpression of some other DREB homologs such as AtCBF3 and AtCBF4 was investigated in transgenic *Arabidopsis* (Gilmour et al. 2000; Haake et al. 2002). Transgenic *Arabidopsis* plants that expressed AtCBF3 showed freezing tolerance, while overexpression of AtCBF4 conferred freezing and dehydration tolerance. Similarly, transgenic *Arabidopsis* that expressed GmDREB2 under both constitutive and stress inducible promoters showed drought and salt stress tolerance (Chen et al. 2007).

Similarly, by using NAC transcription factor, Nakashima et al. (2007) developed transgenic rice overexpressing the stress inducible SNAC1 gene, displayed drought and salt tolerance at the four-leaf stage and had 22–34 % higher seed setting in the field than the negative control under severe drought stress conditions at the reproductive stage. Overexpression of SNAC2/OsNAC6 in rice improved drought and salt tolerance of transgenic plants (Nakashima et al. 2007). In transgenic rice, the Os01g66120/OsNAC2/6 and Os11g03300/OsNAC10 genes were found to enhance drought and salt tolerance (Jeong et al. 2010; Nakashima et al. 2009) and Os03g60080/SNAC1 increased grain yield (21–34 %) under drought stress (Hu et al. 2006). In another study, Hu et al. (2008) found that SNAC2/OsNAC6 transgenic rice plants had significantly higher germination and growth rate than their wild counterparts under high salinity conditions. Song et al. (2011) reported that RNA interference (RNAi) transgenic rice plants with reduced OsNAC5 expression showed less tolerance to abiotic stresses than control plants, whereas overexpression of OsNAC5 enhanced abiotic stress tolerance. OsWRKY45-1 and OsWRKY45-2 were isolated from *japonica* and *indica* rice, respectively (Tao et al. 2009). Both genes were induced by cold stress, but was reduced under drought stress (Tao et al. 2011). Transgenic rice plants overexpressing OsWRKY45-1 showed reduced ABA sensitivity, whereas overexpressors of OsWRKY45-2 displayed increased ABA sensitivity and salt stress tolerance. Expression of the OsTIFY11a gene was induced by cold, drought and salt stresses (Ye et al. 2009). Transgenic rice plants overexpressing Osmyb4 showed enhanced chilling tolerance (Park et al. 2010). Overexpression of OsLEA3-1 in rice improved grain yield under drought conditions (Xiao et al. 2007). Plants overexpressing GmNAC085 show enhanced drought tolerance (Le et al. 2011), whereas the overexpression of GmNAC11 led to increased sensitivity to salt and mannitol stresses (Hao et al. 2011).

Likewise, the expression of DgNAC1, TaNAC2a and EcNAC1 were strongly induced by NaCl and drought stresses in transgenic tobacco plants (Liu et al. 2011a; Ramegowda et al. 2012; Tang et al. 2012). Overexpression of TaNAC2 resulted in enhanced tolerances to drought, salt and freezing stresses in *Arabidopsis* (Mao et al. 2012). The authors reported that gene expression of TaNAC67 was involved in response to drought, salt, cold and ABA treatments. Morphological analysis indicated the transgenics had enhanced tolerances to drought, salt and freezing stresses, simultaneously supported by enhanced expression of multiple abiotic stress responsive genes and improved physiological traits, including strengthened cell membrane stability, retention of higher chlorophyll contents and Na^+ efflux rates, improved photosynthetic potential and enhanced water retention capability. Overexpression of TaNAC67 resulted in pronounced enhanced tolerances to drought, salt and freezing stresses, therefore it has potential for utilization in transgenic breeding to improve abiotic stress tolerance in crops. A list of various TFs and their stress response in transgenic plants is shown in Table 10.4. Overall it can be concluded that TFs seem to play a crucial role in providing tolerance to multiple stresses generally in both an ABA-dependent and independent manner and through respective cis-elements and DNA binding domains. These TFs like

Table 10.4 List of transgenic plants that over-expressed various TFs and stress response of transgenic plants

Family	Gene	Transgenic plants	Stress tolerance	References
bZIP	ABF1	*Arabidopsis thaliana*	Cold	Choi et al. (2000)
	ABF2	*A. thaliana*	Salt, drought	Choi et al. (2000), Kim et al. (2004), Furihata et al. (2006), and Hossain et al. (2010)
	ABF3	*A. thaliana*	Salt, drought	Choi et al. (2000), Kang et al. (2002), and Abdeen et al. (2010)
	ABF4	*A. thaliana*	Drought, salt, cold	Choi et al. (2000) and Kang et al. (2002)
	GmbZIP1	*A. thaliana*, *Triticum aestivum*	Drought and salt	Shi-Qing Gao et al. (2011)
	GmbZIP44	*A. thaliana*	Salinity, freezing	Liao et al. (2008a)
	GmbZIP62	*A. thaliana*	Salinity, freezing	Liao et al. (2008a)
	GmbZIP78	*A. thaliana*	Salinity, freezing	Liao et al. (2008a)
	GmbZIP132	*A. thaliana*	Salinity	Liao et al. (2008b)
	AtbZIP60	*A. thaliana*	Salinity	Fujita et al. (2007)
	Wlipl9	*Nicotiana tabacum*, *T. aestivum*	Freezing	Kobayashi et al. (2008)
	OsABI5	*Oryza sativa*	Salinity	Zou et al. (2008)
	OsbZIP23	*O. sativa*	Drought, salinity	Xiang et al. (2008)
	OsbZIP72	*O. sativa*	Drought	Lu et al. (2009)
	OsAREB1	*O. sativa*	Drought, heat	Jin et al. (2010)
	AREB1	*A. thaliana*	Dehydration	Furihata et al. (2006)
	ABP9	*A. thaliana*	Drought	Zhang et al. (2008a, b)
bHLH	AtMYC2	*A. thaliana*	Osmotic stress	Abe et al. (2003)
MYB	AtMYB2	*A. thaliana*	Osmotic stress	Abe et al. (2003)
	MYB15	*A. thaliana*	Drought, salinity	Ding et al. (2009)
	OsMYB3R-2	*A. thaliana*	Drought, salinity, cold	Dai et al. (2007)
	OsMYB4	*A. thaliana*, *O. sativa*	Freezing	Vannini et al. (2004) and Park et al. (2010)
	OsMYB4	*A. thaliana*	Drought	Mattana et al. (2005)
	OsMYB4	*Lycopersicon esculentum*	Drought	Vannini et al. (2007)
	OsMYB3R-2	*A. thaliana*	Drought, salt, cold	Dai et al. (2007)

CBF/DREB	AtDREB1A	*A. thaliana*	Drought, freezing	Liu et al. (1998)
	AtDREB1A	*N. tabacum*	Freezing, drought	Kasuga et al. (2004)
	AtDREB1A	*T. aestivum*	Drought	Pellegrineschi et al. (2004)
	AtDREB1A	*O. sativa*	Drought, salinity	Oh et al. (2005) and Ravikumar et al. (2014)
	AtDREB1A	*Solanum tuberosum*	Salinity, freezing	Behnam et al. (2006), Celebi-Toprak et al. (2005), and Behnam et al. (2007)
	AtDREB1A	*Arachis hypogaea*	Drought	Bhatnagar-Mathur et al. (2006)
	AtDREB1a	*Chrysanthemum indicum*	Heat stress	Hong et al. (2006)
	AtDREB1A	Tall fescue	Drought	Zhao et al. (2007)
	AtDREB2A-CA	*Arabidopsis*	Drought	Sakuma et al. (2006)
	DREB	*T. aestivum*	Drought	Wang et al. (2006)
	DREB1	*Aloe vera*	Cold	Wang and He (2007)
	DREB1A	*A. hypogaea*, *Lolium perenne*, *Cicer arietinum*	Dehydration, freezing, drought	Vadez et al. (2007), Li et al. (2011), and Bhatnagar-mathur et al. (2007)
	DREB2/DREB3	Wheat and Barley	Drought and frost	Morran et al. (2011)
	TADREB2/TADREB3	*T. aestivum*	Drought	OGTR (2008)
	CBF3	*Hordeum vulgare*, *Arabidopsis*	Cold, drought	Choi et al. (2002) and Li et al. (2011)
	LpCBF3	*Lolium perenne*	Cold	Xiong and Fei (2006)
	AtCBF1	*Arabidopsis*, *S. lycopersicum*	Salinity, water deficit	Jagio-Ottosen et al. (1998) and Hsieh et al. (2002)
	TsCBF1	*Zea mays*	Drought	Zhang et al. (2010)
	AtCBF2	*Lycopersicon esculentum*	Freezing	Hseih et al. (2002)
	AtCBF3	*A thaliana*	Freezing	Gilmour et al. (2000)
	AtCBF4	*A. thaliana*	Freezing	Haake et al. (2002)
	AtCBF1-3	*S. tuberosum*	Freezing	Pino et al. (2007)
	BNCBF5	*Brassicanapus*	Freezing	Savitch et al. (2005)

(continued)

Table 10.4 (continued)

Family	Gene	Transgenic plants	Stress tolerance	References
	BNCBFs 5,7 and 16	*Brassica napus*	Cold	Gao et al. (2002)
	Group I and II DREBs	*B. napus*	Cold	Zhao et al. (2006)
	AtDREB2C	*A. thaliana*	Thermotolerance	Lim et al. (2007)
	OsDREB1A	*A. thaliana*, *O. sativa*	Drought, salinity, Freezing, cold	Dubouzet et al. (2003) and Ito et al. (2006)
	OsDREB1B	*O. sativa*	Drought, salinity, cold	Dubouzet et al. (2003), Ito et al. (2006), and Qin et al. (2007)
	OsDREB2B	*A. thaliana*	Drought, thermotolerance	Matsukura et al. (2010)
	OsDREB1F	*O. sativa*, *A. thaliana*	Drought, salinity, freezing	Wang et al. (2008)
	OsDREB1G	*O. sativa*	Drought	Chen et al. (2008)
	ZmDREB2A	*A. thaliana*, *Zea mays*	Drought, thermotolerance	Qin et al. (2007)
	PgDREB2A	*N. tabacum*	Hyperionic, hyperosmotic salt, osmotic	Agarwal et al. (2010)
	AhDREB1	*N. tabacum*, *Atriplex hortensis*	Drought, salinity	Shen et al. (2003)
	LeCBF1	*thaliana*	Freezing	Zhang et al. (2004)
	GhDREB1	*N. tabacum*	Freezing	Shan et al. (2007)
	CAP2	*N. tabacum*	Drought, salinity	Shukla et al. (2006)
	PeDREB2	*N. tabacum*	Salinity	Chen et al. (2009)
	HvDREB1	*A. thaliana*	Salinity	Xu et al. (2009)
	HvCBF4	*O. sativa*	Drought, salt, chilling	Oh et al. (2007)
	GmDREBa, GmDREBb, GmDREBc	*Glycine max*	Salt, drought, cold	Li et al. (2005)
	GmDREB1	*Medicago sativa*	Salt	Jin et al. (2010)
	GmDREB2	*N. tabacum*, *G. max*	Drought, salt	Chen et al. (2007)
	BjDREB1B	*N. tabacum*, *B. juncea*	Drought, salt	Cong et al. (2008)

	StEREBP1	*S. tuberosum*	Cold, salt	Lee et al. (2007a, b)
	DmDREBa, DmDREBb	*Chrysanthemum indicum*	Cold	Yang et al. (2009a, b)
	FaDREB1	*Festuca arundinacea*	Cold	Tang et al. (2005)
	MbDREB1	*Arabidopsis*	Drought, salt, cold	Yang et al. (2011)
	MtDREB1c	*M. trucatula*	Freezing	Chen et al. (2010)
	DgDREB1A	*A. thaliana*	Drought and salt	Tong et al. (2009)
	HsDREB1a	*Paspalum notatum*	Drought and salt	James et al. (2008)
NAC	AtNAC2	*A. thaliana*	Drought	Tran et al. (2004)
	AtNAC019	*A. thaliana*	Drought	Tran et al. (2004)
	AtNAC055	*A. thaliana*	Drought	Tran et al. (2004)
	SNAC1	*O. sativa*	Drought, salinity	Hu et al. (2006) and You et al. (2013)
	OsNAC5	*O. sativa*	Salinity	Takasaki et al. (2010) and Song et al. (2011)
	OsNAC6	*O. sativa*	Drought, salinity	Nakashima et al. (2007)
	OsNAC10	*O. sativa*	Drought	Jeong et al. (2010)
	ONAC045	*O. sativa*	Drought, salinity	Zheng et al. (2009)
	ONAC063	*O. sativa*	High temperature and high salinity	Yokotani et al. (2009)
	TaNAC2	*A. thaliana*	Drought, salt and freezing	Mao et al. (2012)
	TaNAC67	*A. thaliana*	Drought, salt, cold	Mao et al. (2014)
	TaNAC67	*T. aestivum*	Oxidative stress	Xue et al. (2011)
	TaNAC2a	*N. tabacum*	Drought	Tang et al. (2012)
	TaNAC2a, TaNAC4a, TaNAC6, TaNAC7, TaNAC13, TaNTL5 and TaNAC4	*T. aestivum*	Dehydration, salinity and low temperature	Tang et al. (2012)

(continued)

Table 10.4 (continued)

Family	Gene	Transgenic plants	Stress tolerance	References
	RgNAC2 or RhEXPA4	*R. hybrida*	Dehydration	Dai et al. (2012)
	OsNAP	*O. sativa*	High salinity, Drought and low temperature	Chen et al. (2014)
	CsNAM	*Camellia sinensis*	Drought, Osmoticum, salt, heat and hydrogen peroxide	Paul et al. (2012)
	GmNAC11, GmNAC20	*G. max*, *A. thaliana*	Salt and freezing	Hao et al. (2011)
	GmNAC085	*G. max*	Dehydration	Le et al. (2011)
	DgNAC1	*N. tabacum*	ABA, salt, drought and cold	Liu et al. (2011a, b)
	EcNAC1	*N. tabacum*	Water-deficit and salt stress	Ramegowda et al. (2012)
ERF	SodERF3	*N. tabacum*	Drought, salinity	Trujillo et al. (2008)
	GmERF3	*N. tabacum*	Drought, salinity	Zhang et al. (2009)
WRKY	OsWRKY89	*O. sativa*	Uv-irradiation	Wang et al. (2007a, b)
	OsWRKY45	*A. thaliana*	Drought, salinity	Qiu and yu (2009)
	OsWRKY45-2	*O. sativa*	Salinity	Tao et al. (2009)
	GmWRKY21	*A. thaliana*	Freezing	Zhou et al. (2008)
	GmWRKY54	*A. thaliana*	Drought, salinity	Zhou et al. (2008)
	TaWRKY10	*N. tabacum*	Drought and salinity	Wang et al. (2013)

ZFP	Alfin1	*Medicago sativa*	Salinity	Winicov and Bastola (1999)
	SCOF-1	*N. tabacum*	Freezing	Kim et al. (2001)
	ZPT2-3	*Petunia hybrida*	Drought	Sugano et al. (2003)
	OSISAP1	*N. tabacum*	Drought, salinity, freezing	Mukhopadhyay et al. (2004)
	OSISAP2	*Allium cepa*	Salinity	Xu and Cui (2007)
	Zat12	*A. thaliana*	Oxidative, light stress	Davletova et al. (2005)
	Zat7	*A. thaliana*	Salinity	Ciftci-Yilmaz et al. (2007)
	CaZF	*N. tabacum*	Salinity	Jain et al. (2009)
	ZFP252	*O. sativa*	Drought, salinity	Xu et al. (2008)
	ZFP245	*O. sativa*	Drought, cold	Huang et al. (2009a)
	ZFP179	*O. sativa*	Salinity	Sun et al. (2010)
Others	HARDY	*O. sativa*	Drought, salinity	Karaba et al. (2007)
	AP37	*O. sativa*	Drought	Oh et al. (2009)
	AP59	*O. sativa*	Drought	Oh et al. (2009)
	DST	*O. sativa*	Drought, salinity	Huang et al. (2009b)
	OsTIFY11a	*O. sativa*	Salinity, mannitol	Ye et al. (2009)
	GhDBP2	*Gossypium hirsutum*	Drought, salt, low temperature	Huang et al. (2008)
	OsBZ8	*O. sativa*	Salt	Kakali et al. (2006)
	OCPI	*O. sativa*	Drought	Huang et al. (2007)
	TaSrg6	*A. thaliana*	Drought	Xiang et al. (2007)
	HsfA2	*A. thaliana*	Resistant to environmental stress	Nishizawa et al. (2006)
	ATAF1	*A. thaliana*	Environmental stress	Jensen et al. (2013)

Source: Modified from Lata et al. (2011)

ABRE, MYC/MYB, CBF/DREBs and NAC can be genetically engineered to produce transgenic with higher tolerance to drought, salinity, heat and cold using different promoters. Thus, TFs can be used to develop crop varieties with stress tolerance by genetic engineering approach.

Similar results were also observed using the stress-inducible promoter rd29A in transgenic *Arabidopsis*, tobacco (Kasuga et al. 2004) and rice (Datta et al. 2012). Agarwal et al. (2010) reported that PgDREB2A from *Pennisetum glaucum* is a powerful transcription factor to engineer multiple stress tolerance in tobacco plants. The DREB transcription factors contains conserved ERP/AP2 binding domain bind specifically to DRE/CRT motif and regulate abiotic stress mediated gene expression. The PgDREB2A protein lacks any potential PEST sequence, which is known to act as a signal peptide for protein degradation. The transgenics exhibited enhanced tolerance to both hyperionic and hyperosmotic stresses. At lower concentration of NaCl and mannitol, seed germination and seedling growth was similar in WT and transgenic, however at higher concentration germination in WT decreased significantly. The quantitative real-time PCR of transgenic showed higher expression of downstream genes NtERD10B, HSP70-3, Hsp18p, PLC3, AP2 domain TF, THT1, LTP1 and heat shock (NtHSF2) and pathogen-regulated (NtERF5) factors with different stress treatments. In another study, Mukhopadhyay et al. (2011) studied the transcriptional regulation of genes encoding rice 60S ribosomalprotein L32 (rpL32) in response to salt stress. Northern and RT-PCR analyses showed a significant downregulation of rpL32 transcripts under abiotic stress conditions in rice. Of the four rpL32 genes in rice genome, the gene on chromosome 8 (rpL32_8.1) showed a higher degree of stress-responsive downregulation in a salt sensitive rice variety than in a tolerant one and its expression reverted to its original level upon withdrawal of stress. The nuclear run-on and promoter reporter assays revealed that the downregulation of this gene is transcriptional and originates within the promoter region.

10.7 Conclusions and Prospects

Abiotic stresses adversely affect plant growth and productivity and trigger a series of morphological, physiological, biochemical and molecular changes. In response to these stresses, such as drought, salinity, heat, cold and mechanical wounding, many genes are regulated, and their gene products function in providing stress tolerance to plants. In recent years, several successful attempts have been made to transfer these genes, either singly or in combinations, to confer abiotic stress tolerance in major crops. Compatible osmolytes, ROS scavengers and TFs have received maximum attention from plant scientists and have therefore been targeted for developing abiotic stress tolerant transgenics. Transgenic technologies have shown tremendous potential in producing abiotic stress tolerant plants and pyramiding of important genes have shown superior performance over their individual genes.

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Chapter 11
Breeding Strategies to Enhance Drought Tolerance in Crops

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Abstract Global climate change is expected to increase the occurrence and severity of drought episodes due to increasing temperatures and evapotranspiration. Therefore, food security in the twenty-first century will increasingly depend on the release of new cultivars with improved adaptation to drought conditions. However, selection for drought tolerance is difficult due to a complex genotype and by environment interactions. Recent progress in genomics makes possible a more efficient assessment and enhanced diversity in germplasm collections, introgression of valuable traits from new sources and identification of the genes that control key traits. Marker-assisted selection helps to reduce the environmental impact of breeder selection. Significant advances have been made in the development of in vitro selection methods. The broader use of traits from alien species and the manipulation of heterosis and polyploidy create new perspectives for improving yield potential and adaptation to abiotic stresses. The use of the knowledge generated by these approaches should clarify the functional basis of drought adaptation traits. The integration of these new methods and tools into breeding programs and their potential impact in the development of drought-tolerant germplasm are discussed.

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11.1 Introduction

Water stress occurs when plants are unable to meet evapotranspiration demand. It is induced by unavailability of water due to erratic rainfall or inadequate irrigation, and can be exacerbated by other factors such as soil salinity and physical properties, and high air or soil temperature. Soil salinity makes water unavailable to plants by inducing osmotic stress (Munns 2002). Soil texture and structure determine various properties such as porosity and surface roughness which in turn affect soil holding capacity and water infiltration and retention. Water-stress symptoms appear more rapidly on plants grown in sandy as compared to clay-textured soils (Sullivan 2002). High temperatures also affect water availability by increasing transpiration and water loss (Istanbulluoglu et al. 2009).

Drought is a major production constraint in agriculture worldwide. It is estimated that cultivation is only possible on 16 % of the potentially arable area due to limited water availability (Alexandratos and Bruinsma 2012). Drought is occurring on all continents, with varying intensities and frequencies. The Horn of Africa is strongly affected by drought almost every 12 years but drought intensified in 2009–2011. Around 17 % of the global cultivated area was affected by drought during the period 1980–2006 (Dai 2013). Wheat yield was reduced by 45 % in Kenya in 2009–2011 (Rojas et al. 2011) and by 46 % in Australia in 2006 (van Dijk et al. 2013). Drought principally affects crops cultivated under rainfed conditions, which represent 80 % of the total cultivated area worldwide. According to Pandey et al. (2007), at least 23 million ha of rainfed rice (20 % of the total rice area) in Asia are cultivated under drought-prone conditions. In Pakistan, around 33 % of wheat, 27 % of maize, 56 % of sorghum and millet, 52 % of barley, 77 % of chickpea, 84 % of pulses, 24 % of rapeseed and mustard, and 100 % of castor bean are cultivated under rainfed conditions and are consequently drastically affected by drought (GOP 2014). The part of the cultivated area permanently affected by drought at the world level is estimated to be around 28 % in sorghum, 20 % in wheat, 19 % in barley and 19 % in maize (Li et al. 2009). These percentages vary significantly among regions and years (Table 11.1).

The impact of drought stress on total yield also varies with the region and crop and the occurrence of other stresses like high temperatures (Table 11.2). Pandey et al. (2007) estimated that 36 % of total crop production is lost due to drought in eastern India.

Global climate change increases the occurrence and severity of drought events, in particular because of the increase in evapotranspiration induced by rising temperatures (Feng et al. 2013). Li et al. (2009) estimated that drought prone areas increased by 67 %, 129 %, 55 %, 76 %, 60 % and 70 %, between 1979 and 2006,

Table 11.1 Proportion of the cultivated area affected by drought in different regions and for different crops

Crop species	Region	Proportion of cultivated area affected by drought (year)
Barley (*Hordeum vulgare* L.)	Australia	70 % (1981), 65 % (1997)
Maize (*Zea mays* L.)	South America	50 % (1993)
	Eastern Asia	50 % (2006)
	Europe	60 % (2004)
Rice (*Oryza sativa* L.)	South-East Asia	65 % (1997)
Wheat (*Triticum aestivum* L.)	Africa	80 % (1991)
	North America	47 % (1989)
	South Asia	65 % (2000)

Source: Li et al. (2009)

Table 11.2 Impact of drought stress on yield reduction in various crops

Crop species	Yield reduction %	Geographic location	References
Barley (*Hordeum vulgare* L.)	49–57	Jordan	Samarah (2005)
Castor bean (*Ricinus communis* L.)	60	Pakistan	Cheema et al. (2013)
Chickpea (*Cicer arietinum* L.)	50–80	France	Leport et al. (1999)
	15	Pakistan	Bakhsh et al. (2007)
Common bean (*Phaseolus vulgaris* L.)	78	Romania	Szilagyi (2003)
Groundnut (*Arachis hypogea* L.)	55–72	India	Hamidou et al. (2013)
	22	Pakistan	Naeem-ud-Din et al. (2012)
Maize (*Zea mays* L.)	48	Czech Republic	Hlavinka et al. (2009)
	43–80	Iran	Khalili et al. (2013)
Oat (*Avena sativa* L.)	79	Czech Republic	Hlavinka et al. (2009)
Pearl millet (*Pennisetum glaucum* (L.) R. Br.)	25	Pakistan	Saifullah et al. (2011)
Potato (*Solanum tuberosum* L.)	89	Czech Republic	Hlavinka et al. (2009)
Rape (*Brassica napus* L.)	39	Czech Republic	Hlavinka et al. (2009)
Rice (*Oryza sativa* L.)	42–66	India	Raman et al. (2012)
	65	Thailand	Jongdee et al. (2006)
Rye (*Secale cereal* L.)	52	Czech Republic	Hlavinka et al. (2009)
Sorghum (*Sorghum bicolor* (L.) Moench)	17	Pakistan	Malik et al. (2007)
Wheat (bread) (*Triticum aestivum* L.)	57	Czech Republic	Hlavinka et al. (2009)
	6–39	Serbia	Denčić et al. (2000)
Wheat (durum) (*T. durum* Desf.)	74	Turkey	Kiliç and Yağbasanlar (2010)

for barley, maize, rice, sorghum, soybean and wheat, respectively. Drought stress is expected to be more severe in the coming years and drought affected areas may double by 2050. The most affected areas of the world may include North and Central America, Southern China and Southern Brazil. The erratic and unpredictable nature of the drought and floods cycles will also increase (Douglas et al. 2008).

Drought can have a tremendous economic impact. Drought faced by the US Great Plains grain belt in 2012 pushed up world food prices by 3–4 % in 2013 (Yuan and Quiring 2014). Food security in the twenty-first century will consequently increasingly depend on the release of cultivars with improved tolerance to drought conditions and with high yield stability (Pennisi 2008).

This chapter discusses screening and breeding methods with emphasis on bridging the gap between genotype and phenotype. Some examples of success in the development of drought tolerant germplasm are presented.

11.2 Empirical Breeding vs. Analytical Breeding for Drought Tolerance

There are two main approaches to improving economic yield, i.e. the empirical approach in which the plant breeder directly selects the breeding material for yield per se and the analytical approach which emphasizes the improvement of yield through indirect selection for morphological, physiological or biochemical traits associated with yield.

The empirical approach results in the development of a plant population adapted to specific drought conditions and should be done in the target environment. Yield has a low heritability and therefore selection based on this complex trait is difficult and brings very slow improvement. After having used yield under drought as an exclusive breeding objective, most breeders have progressively replaced this empirical approach by indirect selection (Jackson et al. 1996) based on the selection for *secondary traits* or plant characteristics that provide additional information about how the plant performs under a given environment (Lafitte et al. 2003). The selection criteria used in indirect selection should show relationships with yield under drought environment. Narrow-sense heritability of the trait(s) of interest should be high so that breeding populations show good response to selection. Moreover, trait(s) should be, as far as possible, easily measurable and not plant destructive (Edmeades et al. 1997).

A good example of indirect selection is offered by the improvement of drought tolerance in tropical maize at the International Center for Wheat and Maize Improvement (CIMMYT), in Mexico. Bolaños and Edmeades (1996) noted significant phenotypic correlations between yield and ears plant^{-1}, kernels plant^{-1}, anthesis-silking interval (ASI), leaf rolling and leaf senescence. Genetic variances of yield contributing traits generally decreased with the intensity of water stress. However, traits such as anthesis-silking interval and kernel spike^{-1} showed an increase in

genetic variance. Heritability estimates for these traits were higher than for yield, making them useful for indirect selection. Indirect selection for yield in populations with a broad genetic basis resulted in significant drought tolerance improvement (Edmeades et al. 1999). Yield gain was 0.08–0.26 mg ha^{-1} $cycle^{-1}$ in three populations under drought stress. There was a positive gain in harvest index (0.025 $cycle^{-1}$) showing that yield gains were due to better photosynthates mobilization to ears under drought stress. Chapman and Edmeades (1999) noted a significant increase of ear $plant^{-1}$, grain $plant^{-1}$ and a reduction of ASI and senescence for each cycle of recurrent selection. The effectiveness of indirect selection was confirmed by Monneveux et al. (2006). After 6–9 cycles of selection in the source populations DTP1 and DTP2, there was significant increase in grain yield and a significant reduction in ASI and in the abortion rate of ovules. Quantitative trait loci (QTLs) have been identified for these traits (Hao et al. 2008) and marker-assisted selection (MAS) developed (Ribaut and Ragot 2007).

11.3 Evaluation for Drought Tolerance

11.3.1 Phenotyping: The Main Bottleneck in Breeding for Drought Tolerance

Plant phenotyping (from the Greek *phainein*, to show) is the basic measurement of individual quantitative parameters that form the basis for more complex traits such as growth, development, architecture and yield. Plant phenotyping has been performed by farmers since crop domestication began and by breeders over the last century. Over the last two decades, efforts were made to develop more reproducible measurements. The basic attributes of good phenotyping are not just the accuracy and precision of measurements, but also the relevancy of experimental conditions. Efficient phenotyping implies accurate (i) definition of the target environment, (ii) characterization of the managed stress (testing) environment, (iii) stress monitoring and (iv) measurement of secondary traits. The different approaches and tools that are required to perform correct phenotyping in different crops are summarized in Monneveux and Ribaut (2011).

11.3.2 Target Environment

Any variety is adapted to several environments. This group of environments is referred to by Fischer et al. (2003) as *target population of environments* (TPE). Deploying different cultivars in different TPEs allows reducing genotype by environment interactions (GEI). An important objective for breeders is to clearly define the TPE for which each variety is developed. The definition of the TPE can be performed based on the

identification of mega-environments, genotype by environment interaction analysis, spatial analysis and modeling.

A first step in the definition of the TPE is the identification of mega-environments (ME) or agroecological zones, mainly based on spatial information about environmental constraints including drought (Rajaram et al. 1995). This requires information about crop distribution, environmental constraints and the factors to which the crop is susceptible. However, the ME and agroecological zones do not always offer a sufficient level of resolution in the definition of the TPE.

An important objective by implementing ME and analyzing GEI is, besides describing the behavior of genotypes across different environments, to define clusters of locations sharing the same best cultivar(s), i.e. showing little or no crossover (Yan and Rajcan 2002). Biplot analysis and AMMI (additive main effects and multiplicative interaction) and GGE (genotype main effects and genotype by environment interaction effects) models have been used in several crops for clustering locations and defining TPE (Yan et al. 2007). Because of the non-predictable component of GEI associated with year-to-year variation, substantial datasets (20 or more varieties evaluated) and multi-year evaluations are required to accurately estimate the best clustering (Cooper et al. 1999). The high temporal variability of climatic variables can be addressed using long-term historical (Loffler et al. 2005) or simulated/predicted climatic records (CCAFS 2011).

Recent progress in the development of computer hardware and software, and the availability of soil and climate data in digital formats, have allowed sophisticated statistical analysis of GEI (Crossa et al. 2004), development of precise agroecological zoning maps (Hyman et al. 2014) and classification of locations into more or less homogenous environment types (Roozeboom et al. 2008). Linking individual trial sites to larger regions for which they are representative is very useful for developing TPE maps and, ultimately, for introducing varieties into environments where they are expected to perform well (Gauch and Zobel 1997).

In cereals a great advance in the study of TPE was attained using long-term climatic records as an input of crop growth models (Chenu et al. 2011). This approach permits identification of model parameters, to run the model under different climatic scenarios and to test it in the multi-environment trials (Tardieu 2012).

11.3.3 Managed Stress Environment

As breeding facilities (fields, equipment) are generally not available in the TPE and genetic resources cannot be easily transferred, phenotyping needs to be done in a managed stress environment (MSE). Ideally, the MSE should be representative of the TPE with regard to edaphic and climatic conditions (Gomide et al. 2011). Any deviation may result in significant GEI between TPE and MSE, and genetic gains achieved in the MSE may not be expressed in the TPE. Geographic information system (GIS) tools (e.g. homology maps) and models, considerably help in describing the relationships between TPE and MSE (Hyman et al. 2014).

11.3.3.1 Environment Characterization

Quantifying evapotranspiration and crop water requirements in the MSE is essential to control the different water treatments and estimate the corresponding crop stress levels. Atmospheric parameters like air temperature, global solar radiation, relative humidity, wind speed, water vapor pressure deficit and precipitation should be registered. Soil characterization is equally important as soil depth, water-holding capacity, compaction, aluminum toxicity or soil acidity can affect water availability and the imposition of the accurate stress (Gomide et al. 2011). Water balance models such as AQUACROP (FAO 2013) are useful to determine the patterns of water supply and the type of drought (intensity, timing) faced by plants in the MSE.

11.3.3.2 Confounding Factors and Field Homogeneity

The crop facing drought in the field simultaneously experiences a number of additional stresses (e.g. micronutrient deficiency, soil compaction, salinity and pathogens) that impair root growth, reduce water availability, exacerbate the effects of the water stress and bias an accurate evaluation of its impact. Spatial variability becomes more apparent under drought (Gomide et al. 2011) and affects the detection of treatment differences by inflating the estimated experimental error variance (Masuka et al. 2012). Direct assessment of soil variability within a field can be made through soil sampling positioned by a Global Positioning System (Campos et al. 2011). High throughput techniques are also available for mapping variability within field sites, based on penetrometers (Cairns et al. 2011), soil electrical conductivity and electromagnetic induction sensors (Cairns et al. 2012), spectral reflectance (Dang et al. 2011) and thermal imagery of canopy (Campos et al. 2011).

11.3.3.3 Stress Application

The ability to manage timing and intensity of the stress in the MSE is a key factor in mimicking the environmental conditions prevailing in the TPE (Tuberosa 2014). An increasing number of breeding programs are conducting drought trials in dry locations, or *out-of-season*, i.e. in seasons that are not the cropping season of the crop and are characterized by very low rainfall. Under such conditions the dynamics of drought episodes can be tightly controlled through the frequency and volume of irrigation treatments. In the case of out-of-season experiments, the dry season has to be sufficiently long to cover the whole growth cycle and may reflect the environmental conditions the crop would experience during a natural drought in the main (wet) season (Jagadish et al. 2011). Different radiation, temperature and vapor-pressure deficit lead to genotype-by-season interactions and limit the extrapolation of results. Late or delayed planting can represent an interesting alternative option.

To apply a similar drought stress (in terms of timing and intensity) in the MSE as experienced in the TPE, irrigation should be applied at the correct phenological stage. Genotypes with different phenologies might face different stress durations

biasing the interpretation of the influence of drought-adaptive traits on yield (Tuberosa 2014). To overcome this difficulty, genotypes can be grouped into subsets of similar earliness to ensure phenological synchronization across genotypes at the stage when water stress is imposed. Another option is to use earliness as a covariate adjustment.

Finally, irrigation methods must be carefully chosen to ensure optimal control of the irrigation water (Gomide et al. 2011). The accurate management of irrigation requires an adequate characterization and monitoring of soil and plant water status. Jones (2007) highlighted that over half of the published papers focusing on the effects of drought on gene expression or transgenes did not include measurement of plant or soil water status. Soil or plant water status can be monitored by measuring water potential (Blum 2009) or relative water content (Riga and Vartanian 1999). Methods for measuring the amount of water stored in the soil include the gravimetric method, polymer-based tensiometer (van der Ploeg et al. 2008), neutron probe (Hignett and Evett 2008), capacity probe (Nagy et al. 2008), time-domain reflectometry (Noborio 2001), single and multi-sensor capacitance probe systems (Fares and Polyakov 2006) and two dimensional geo-electrical tomography (Werban et al. 2008).

11.3.3.4 Statistical Designs

The effectiveness of field experiments and the management and interpretation of phenotypic data depend on the utilization of appropriate experimental designs to allow control within-replicate variability and reduce or remove spatial trends (Federer and Crossa 2011). A randomized complete block design (RCBD) is useful when the number of genotypes is small and the field gradient is gradual. Incomplete block designs are used when the number of accessions is large and heterogeneity is high within the block. Alpha-lattice design is a type of incomplete block design which can accommodate any number of accessions and replication. Augmented design is a type of incomplete block design which can accommodate any number of accessions in a single replication. Checks are replicated in all sub blocks and are used to calculate the error terms.

The accurate phenotyping of large numbers of plots is facilitated by high throughput experimental machinery (e.g. plot combines able to measure yield directly in the field) and automation of tedious manual operations. The labeling of plots and samples, data collection and storage are facilitated by the use of bar-coding and dedicated software (e.g. spreadsheets and databases).

11.3.3.5 Controlled Environments

Static or moveable rainout shelters constitute an alternative to protect the experiment from rainfall and investigate the adaptive response of crops to a desired level of drought stress (Tuberosa 2014). A major inconvenience is, in addition to the construction and operating costs, the rather limited area protected which reduces the number and size of experimental plots.

Phenotyping potted plants in greenhouses or growth chambers with robotized systems and advanced image analysis software to assess traits in a quicker and more reproducible manner represents an interesting option for the analysis of underlying drought mechanisms (Fiorani and Schurr 2013). However, the differences between controlled conditions and those prevailing in the TPE may limit the application of results in germplasm development. In particular, irrigation in pots creates a situation that is very distinct from that occurring under field conditions. Potted plants are exposed to earlier and stronger stress (Wahbi and Sinclair 2005) and the temperature of the substrate is generally different from that of field soil (Passioura 2005).

11.4 Assessment and Measurement of Traits

The heritability of indirect traits varies according to the genetic make-up of the materials under investigation, the conditions under which the materials are evaluated and the accuracy and precision of the phenotypic data. The accuracy of secondary traits measurement is closely related to their repeatability (Tuberosa 2014).

11.4.1 Phenological Traits

Days from sowing (or emergence) to flowering and maturity are often used to evaluate earliness, the main trait for drought avoidance. As mentioned previously (Sect. 11.2), a valuable selection target for improving drought adaptation in maize is provided by the anthesis-silking interval (ASI), a trait that is usually negatively correlated with grain yield under drought conditions, has an intermediate heritability and can be phenotyped easily (Monneveux and Ribaut 2006).

11.4.2 Early Vigor

Early vigor has been used to improve water-use efficiency (WUE) and yield in wheat (Rebetzke et al. 2007). Spectral reflectance is increasingly used to estimate early vigor (Montes et al. 2007). QTLs for the growth rate of wheat seedlings have been identified (Spielmeyer et al. 2007).

11.4.3 Root Traits

The main obstacle in using root traits as selection criteria relates to the difficulty of phenotyping them in field-grown plants (Richards 2008). Excavation and coring methods, although cumbersome, have often been used to estimate root mass and

distribution (Nissen et al. 2008). The vertical pulling strength required to uproot the plant has been proposed as a proxy for root mass and architecture (Landi et al. 2002). A high throughput, albeit also destructive technique known as *shovelomics*, has been recently deployed to investigate several root traits in field grown maize (Trachsel et al. 2011). Minirhizotrons provide a non-destructive, in situ method for directly studying fine roots (Smit and Groenwold 2005). Tube installation is critical, and can lead to soil disturbance and the creation of artifacts (Johnson et al. 2001). In maize, a fast and non-destructive estimation of root mass has been performed using a hand-held capacitance meter (McBride et al. 2008). Heterogeneity in soil structure has limited the use of this technique.

Root traits have been often measured under hydroponics or aeroponics and controlled conditions (Ren et al. 2012). A major weakness of these techniques is the artificial environment in which the roots grow, limiting the extrapolation of results to field grown plants. In maize, a significant positive association has however been reported between seminal root traits in hydroponics and root pulling resistance in the field (Tuberosa et al. 2002).

Growing plants in pots, columns or observation chambers filled with soil (Zaman-Allah et al. 2011) permits measurement of the amount of water provided to the plants, and an estimation of water use, water use efficiency (Price et al. 2002) and root penetration capacity (Acuna et al. 2007).

Gel- or soil-filled chambers, soil sacs, pouches, paper rolls, X-ray microtomography, and magnetic resonance imaging (MRI) have also been used to investigate bi- and tri-dimensional root architecture (Mace et al. 2012). These techniques are particularly attractive for the identification of QTLs (Tuberosa 2014).

11.4.4 Leaf Rolling

Leaf rolling is an important component of dehydration avoidance. It can be observed in crops such as maize, wheat, rice and sorghum (Fig. 11.1a).

Leaf rolling reduces leaf area thus decreasing transpiration under drought stress. It is however an indicator of reduced turgor, a consequence of poor osmotic adjustment. It affects yield as it reduces the light interception by the canopy. Genotypic differences were observed for leaf rolling and found suitable for screening under mild drought stress. Leaf rolling can be easily scored visually (O'Toole and Cruz 1980).

Singh and Mackill (1991) evaluated F2 and F3 wheat segregating populations developed from a cross between tolerant and susceptible parents and found that leaf rolling was under monogenic recessive control. In rice, several genes related with leaf rolling (RL1 to RL9) were identified. A gene symbolized as NLR1 was mapped on chromosome 12 (Hu et al. 2010). Mutants of this gene showed reduction in leaf width, moderate leaf rolling and reduction in plant height. A mutant gene NAL7 was isolated which caused rolling of leaves and reduction in leaf width (Fujino et al. 2008).

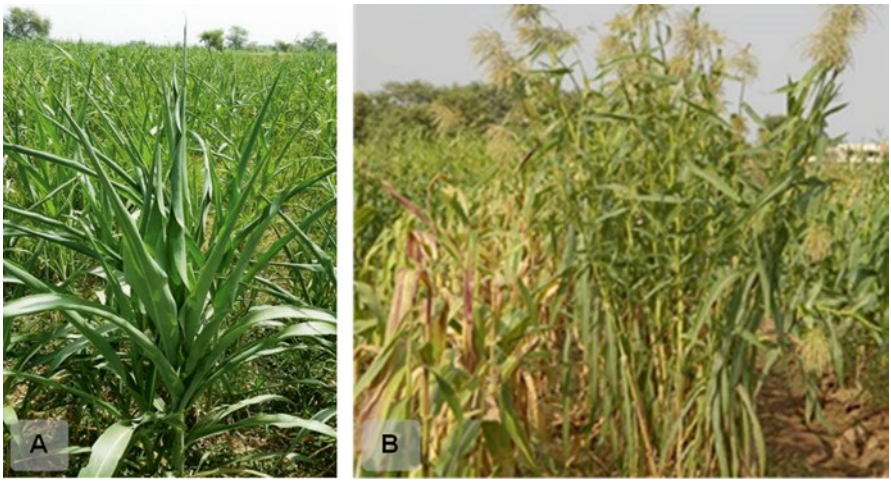

Fig. 11.1 **a** Leaf rolling characteristic of *Sorghum bicolor* (L.) Moench × *Sorghum sudanense* (Piper) Stapf. hybrid under drought stress, **b** Comparison of stay green in maize, *Zea mays* ssp. *mays* (*left*) and teosinte, *Zea mays* ssp. *mexicana* (Schrad.) H.H. Iltis (*right*) (Photos by: S. Rauf)

Bulliform cells which are adaxial cells in the epidermis of the leaf are responsible for leaf rolling. The rice mutant NAL7 has small bulliform cells (Zhang et al. 2009). In some species, leaf rolling also can be due to large hypodermis cells shrinking due to water loss (Kadioglu et al. 2012).

11.4.5 Stay-Green

Stay-green is a post flowering dehydration tolerance mechanism. Leaf senescence reflects a loss of chlorophyll which reduces photosynthesis rate. A well-sustained source capacity is a key factor to maintain yield, particularly in drought-stressed crops. Genotypes with delayed leaf senescence increase cumulative photosynthesis over the crop life cycle (Vadez et al. 2011). The traits monitored most frequently to obtain indirect estimates of photosynthetic potential are chlorophyll concentration, stay-green and delayed senescence, which are interconnected (Shukla et al. 2004). Breeding lines possessing the stay-green trait tend to remain green for longer period. At cell level they maintain the integrity of chloroplast proteins such as LUCP2, OEC33 and Rubisco (Borell et al. 2001).

Grain yield has been reported to be positively related to green leaf area under terminal drought and negative relationships were observed between grain yield and visually scored leaf senescence (Borell et al. 2000a). A yield advantage of 47 % has been reported under terminal drought in stay-green sorghum genotypes (Borell et al. 2000b). Stay-green can be however *cosmetic* (not functional). Some stay-green maize lines, for example, delay leaf senescence under drought stress for

5 days but their photosynthetic rate decreases as compared to lines not having stay-green property. Sometimes stay-green is related to late maturity. This was observed in a comparison between maize and teosinte (Fig. 11.1b). The teosinte stays green for a longer period of time because of its late maturity. The stay-green trait of teosinte can be used to expand fodder supply under heat and drought stress.

Visual evaluation of senescence is considered a useful method for evaluating stay-green under drought stress, even if individual bias can exist among observers for rating. Xu et al. (2000) reported a high correlation between visual rating and chlorophyll content estimated through SPAD measurements. Borell et al. (2000a) found a significant correlation between the visual rating for stay-green and green leaf area at maturity in sorghum species.

Stay-green traits in maize correlate closely to grain yield and stay-green QTLs overlap with yield QTLs (Zheng et al. 2009). In sorghum stay-green is associated with maintenance of a more favorable water status, related to root traits (Mace et al. 2012). Four major QTLs that control stay-green and grain yield have been identified (Harris et al. 2007) and near isogenic lines (NILs) for these QTLs have been generated, allowing a detailed analysis of stay-green physiology and positional cloning of the underlying genes (Vadez et al. 2011).

11.4.6 Canopy Temperature Depression (CTD)

Canopy temperature depression (CTD), the difference in temperature between the canopy surface and the surrounding air, is an index of canopy cooling and transpiration used to discriminate accessions for drought tolerance under dry conditions. CTD is a highly integrated character resulting from the effects of several traits acting at the root, stomata, leaf and canopy levels. It also informs about deep root penetration as genotypes with a cooler canopy temperature under drought stress (higher CTD) use more of the soil available water (Ludlow and Muchow 1990). Infrared thermometry can report subtle differences in leaf temperature in both field and controlled conditions. The trait has a moderate heritability and shows positive relationship with yield (Winterhalter et al. 2011a, b). Measurements are fast and non-destructive but should be made well before maturity, on recently-irrigated crops and on cloudless and windless days with high vapor pressure deficits (Reynolds and Pfeiffer 2000). Significant genetic gains in yield have been reported in response to selection for CTD, mainly in hot and dry environments (Brennan et al. 2007). Grant et al. (2006) confirmed the robustness and sensitivity of thermal imaging for detecting changes in CTD, stomatal conductance and leaf water status in a range of plant species (grapevine, bean and lupin).

In order to estimate the type of genetic variability associated with CTD in sunflower, Rauf et al. (2011) used F2 populations derived from crosses between low

and high CTD inbred lines. All crosses were submitted to two moisture regimes (drought stress and non-stress) in a large pot experiment. CTD was estimated at anthesis using an infra-red thermometer. A preponderance of non-additive gene action with low narrow sense heritability was noted in all crosses. Recurrent selection was practiced for the improvement of CTD. F2 plants were divided into six groups on the basis of variation in CTD (Fig. 11.2). Plants showing higher CTD were selected and intermated in all possible combinations. A broad variation was noted in the next generation showing no evidence of a loss of genetic variation due to recurrent selection. Overall means of the groups increased due to recurrent selection (17, 23 and 16 % in the second, third and fourth cycles, respectively) and differences between groups broadened with each cycle of selection. Recurrent selection for CTD had a positive impact of pollen fertility, stomatal conductance, transpiration, photosynthetic rate and achene oil content and negative impact on water use efficiency (Table 11.3).

11.4.7 Osmotic Adjustment Indicators

Osmotic adjustment (OA) is generally estimated by calculating the difference between osmotic potential under non-stress and drought stress, after rehydration (Babu et al. 1999). The association between OA and productivity varies among crop species and stress intensities. In many cases OA is related to plant survival rather than productivity. Sorghum cultivars with high OA showed a yield advantage of 11 % over low osmotic adjustment genotypes (Morgan et al. 1991). Similarly, a positive relationship was observed between OA and grain yield in chickpea and sunflower (Rauf and Sadaqat 2008; Rauf et al. 2010). Osmotic adjustment is a complex trait and its genetic analysis has been carried out under diverse environments. Teulat et al. (1998) reported a broad sense heritability ranging of 0.04–0.44.

11.4.8 Excised Leaf Water Loss

Excised leaf water loss is determined after removing a leaf of the plant and measuring the decline in fresh weight over time (McCaig and Romagosa 1989). Experiments should be conducted under optimal conditions of temperature, light and humidity. Variation within germplasm for leaf water loss arises due to residual transpiration (i.e. the sum of transpiration through stomata and epidermal layer). Excised leaf water loss relates to drought avoidance rather than drought tolerance. In some species the deposition of cuticle waxes reduces epidermal transpiration (Cameron et al. 2006).

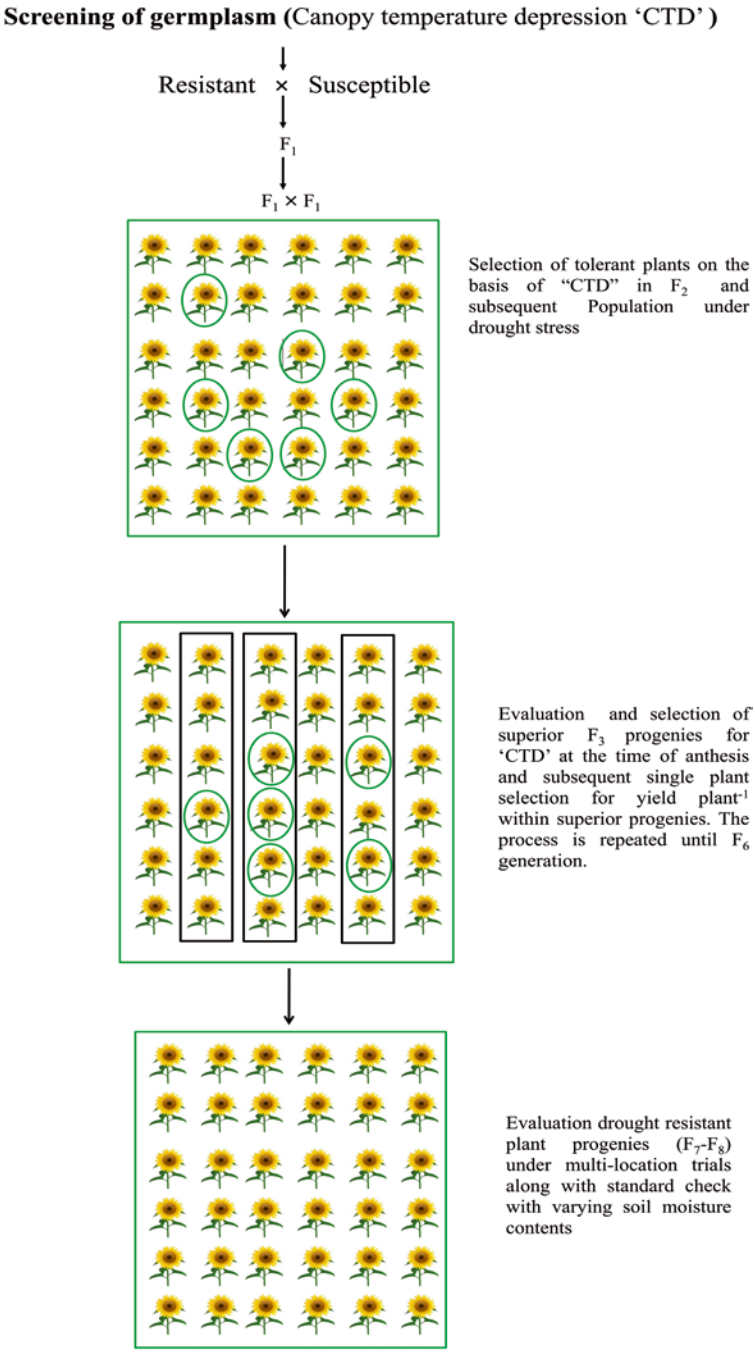

Fig. 11.2 Selection based on canopy temperature depression CTD in sunflower (*Helianthus annuus* L.) (Source: Rauf et al. (2011))

Table 11.3 Mean of various classes of canopy temperature depression (CTD) and corresponding effects on pollen fertility (PF), unfilled grain (UF), stomatal conductance (g_s), net photosynthesis rate (P_N), transpiration rate (T_r), water use efficiency (WUE_i) and seed oil content under drought stress in the unselected F2 base population derived from the cross CM-205 × RL-27 and after the 4th recurrent selection cycle

Classes	CTD (°C)	PF (%)	UF (%)	g_s (mol m^{-2} s^{-1})	P_N (μmol $^{-1}$)	T_r (mol m^{-2} s^{-1})	WUE_i	Oil (%)
Base population (F_2)	0.52 ± 1.66	49.37 ± 32.25	48.13 ± 24.36	365.22 ± 183.29	3.93 ± 2.89	3.48 ± 1.74	1.13 ± 1.29	26.54 ± 4.56
After 4th recurrent cycle								
Very low	0.08 ± 0.03	34.37 ± 4.38	82.41 ± 7.34	89.38 ± 16.35	1.71 ± 0.12	1.18 ± 0.19	1.45 ± 0.14	22.58 ± 1.38
Low	0.31 ± 0.11	34.51 ± 6.38	61.51 ± 11.37	112.44 ± 8.39	1.86 ± 0.29	1.44 ± 0.26	1.29 ± 0.17	26.39 ± 2.19
Medium	0.71 ± 0.10	56.67 ± 4.87	59.71 ± 8.64	316.87 ± 13.51	3.58 ± 0.24	3.12 ± 0.33	1.15 ± 0.16	32.15 ± 1.74
Medium	1.46 ± 0.18	64.39 ± 6.48	44.84 ± 5.81	463.27 ± 16.34	4.58 ± 0.18	3.98 ± 0.27	1.15 ± 0.12	34.15 ± 1.88
High	2.72 ± 0.15	69.58 ± 5.41	26.27 ± 4.71	515.39 ± 9.61	6.54 ± 0.51	6.12 ± 0.63	1.07 ± 0.16	36.04 ± 1.29
Very high	4.92 ± 0.21	78.38 ± 4.13	18.36 ± 5.33	663.58 ± 15.61	8.11 ± 0.42	7.96 ± 0.32	1.02 ± 0.17	36.54 ± 1.62

Source: Principle author (unpublished data)

11.4.9 Analysis of Water Soluble Carbohydrates

In cereals, the efficiency of remobilization of water-soluble carbohydrates (WSC) from the stem and leaves highly contributes to yield in the case of post-anthesis drought (Araus et al. 2002). Rebetzke et al. (2008b) phenotyped three wheat mapping populations for WSC mass per unit area. Lines with high values for this trait produced more fertile tillers associated with greater biomass, grain number and yield.

11.4.10 Digital Imaging

Digital imaging allows analyzing canopy characteristics (Fiorani et al. 2012) through a dynamic, inexpensive and non-destructive visualization of crop growth (White et al. 2012). Digital imaging can also be used for measuring root characteristics at high resolution scales (Blouin et al. 2007), an important prerequisite to investigate the kinetics of root growth (Armengaud et al. 2009).

11.4.11 Remote Sensing

Remote sensing is defined as the set of techniques which allow collecting information about an object without having physical contact with it. Remote sensing methods as near-infrared spectroscopy and spectral reflectance of plant canopies are promising components of high throughput phenotyping (Montes et al. 2007) and provide interesting opportunities for assessing integrative traits with a high temporal resolution (Gutierrez et al. 2010). Remote sensing can detect changes in leaf reflectance and emittance according to leaf thickness, age, pigment composition and water and nutrient status (Hatfield et al. 2008). Various vegetative indices can be calculated based on this information to quantify agronomic parameters (e.g. crop cover, biomass and yield). Spectral reflectance can be used to monitor the presence of different types of stress in plants (Suárez et al. 2009). To facilitate remote-sensing measurements, small and light cameras and radiometers can be mounted on handheld devices (Casadesus et al. 2007) or transported by tractors (Montes et al. 2007) or unmanned aerial vehicles (UAV).

11.4.12 Carbon Isotope Discrimination

Atmospheric CO_2 comprises two stable isotopes, ^{13}C and ^{12}C. The ratio ^{13}C:^{12}C is around 1:99. Plant species are able to discriminate isotopes during CO_2 uptake. Carbon isotopes can be measured in different plant samples such as leaf, stem or

grain using a mass spectrometer. The isotopic composition of a sample is expressed as $\delta 13C(‰) = \left[\left(R_{sample} / R_{standard} \right) - 1 \right] \times 1000$, where $R_{standard}$ is the isotope ratio of a given standard (marine limestone from Pee Dee Cretaceous belemnite formation, South Carolina or an artificial version from Vienna, VPDB). Carbon isotope discrimination (Δ) measures the ratio of stable carbon isotopes (13C/12C) in the plant dry matter compared to the ratio in the atmosphere (Condon et al. 1990). Its value is around 20‰ in C3 plants and 5‰ in C4 plants (Farquhar et al. 1989). The $\delta^{13}C$ values are converted to discrimination values, using the following equation: $\Delta(‰) = \left(\delta^{13}C_{air} - \delta^{13}C_{sample} \right) / \left(1 + \delta^{13}C_{sample} + 1000 \right)$ (Farquhar and Richards 1984), assuming that the $\delta^{13}C$ of air on the site is −8‰ on the PDB scale (Mook et al. 1983).

In C3 crops like wheat, the correlation between Δ and final grain yield varies from negative, when ample water is available to the crop, to negative in drought conditions (Condon et al. 2004). This is due to the influence of both stomatal conductance and photosynthetic activity on Δ (Turner 1997). The association between Δ and yield consequently depends on the environmental conditions, the phenology of the crop and the plant organ (e.g. leaf or grain) from which the samples are collected (Monneveux et al. 2005). A negative correlation also has been noted in C3 crops between Δ and WUE_i (the photosynthetic or intrinsic or instantaneous water-use efficiency, defined as the ratio of the rate of carbon assimilation to the rate of transpiration), allowing an integrated measure of this last trait (Johnson et al. 1990).

A high genetic variation for grain Δ has been reported in C3 species (Chen et al. 2012). This trait has a high heritability (e.g. 0.76–0.85 in durum wheat, Merah et al. 2001) and a low GEI (Rebetzke et al. 2008a). The high cost required to measure each sample makes Δ an interesting candidate for marker-assisted selection (MAS).

11.4.13 Chlorophyll Fluorescence

In addition to reflected energy, a small fraction of the energy absorbed by plants is emitted as chlorophyll fluorescence the dynamics of which are related to changes in the photochemical conversion. Chlorophyll fluorescence is a sensitive indicator of water and heat stress (Flexas et al. 1999) and commercial instrumentation exists for its assessment at leaf-level in the laboratory (Schreiber et al. 1994). Its assessment in open field conditions and at the canopy level is still a challenge. Works such as those by Zarco-Tejada et al. (2009, 2012) suggest the possibility of using the discrimination method of Fraunhofer lines in the absorption bands of atmospheric oxygen (Moya et al. 1998), to remotely obtain chlorophyll fluorescence images providing information at different levels of integration, from a leaf to the whole canopy (Moya and Cerovic 2004).

11.5 Drought Adaptation Improvement

11.5.1 Genetics of Drought Adaptation

Drought adaptation in plants refers to yield stability under water deficit. The genetic control of abiotic stress adaptation is not only very complex, but also highly influenced by environmental factors and the developmental stage of the plant. Inheritance of drought-adaptation traits is important for developing effective breeding schemes. Additive variance which results from the cumulative effect of minor genes, or from their interaction, is selectable through simple breeding procedures such as mass selection or pedigree selection. Interaction among alleles and genes also gives rise to dominance and epistatic effects. Dominance variance is the deviation of heterozygote genotypes from the average effect of the parents, while epistatic variance is due to complex interaction. Dominance and epistasis are the causes of heterosis in cross-pollinated species. Both variances are not selectable in segregating generations and plant breeders use the narrow-sense heritability (additive variance/phenotypic variance) to estimate the proportion of selectable variation from the total variation.

Selection response is the improvement in mean value of a trait due to selection and is dependent on the magnitude of additive variance or narrow-sense heritability and selection intensity. Selection differential is the change in the mean of selected plants to the overall mean of the population. Traits with high narrow-sense heritability are more likely to show good selection response. Realized heritability, the ratio of selection response to selection differential, is another index of selection used to measure the degree to which a trait in a population can be improved by selection.

11.5.2 Introduction of Drought-Adapted Species

Generally, species in very arid climates are well adapted to drought stress and may be recommended to increase area under cultivation and fulfill human needs. Species in the phase of domestication or semi-domesticated species such as some forages show high genetic variation for drought adaptation and may represent potential candidates for introduction in drought prone areas. Some forage legumes (e.g. *Lotus* genus) have been used as model species for a better understanding of drought-adaptation mechanisms (Sanchez et al. 2012). Ashraf (2006) noted that grass species such as *Cenchrus penisetiformis* Hochst.et Steud. ex Steud.; kallar grass (*Leptochola fusca* (L.) Kunth); *Panicum turgidum* Forssk and *Pennisetum divisum* (Gmel.) Henr., adapted to desert conditions of Pakistan, have diverse mechanisms of drought adaptation.

Species of the cactus family (Cactaceae), native of the deserts, have great potential for cultivation as food and feed. Due to their pathways of CO_2 fixation, they are highly water-use efficient. They store water within their swollen stem and

pads and have developed specialized structures such as spines, hair and waxes to reduce transpiration losses. The cactus family comprises 30 genera and 1,500 species, including economically-beneficial species of the genera *Opuntia* and *Nopalea*, distributed in various regions of the world (Russel and Felker 1987). A broad genetic variation exists among species for heat, drought and salinity adaptive traits and for fodder yield and fruit characteristics (Barbera 1995). *Opuntia* cultivars bear sweet tasty fruits that are marketed in United States, Mexico, Brazil, North Africa and several European countries. Pads of the spineless *Opuntia* and *Nopalea* species can be consumed directly as fresh green fodder by animals. Spine character is single gene controlled and the spineless character evolved due to a single gene mutation. The fruit yield potential of *Opuntia* range is 8–12 mt ha^{-1} while its dry fodder yield ranges from 20 to 50 mt ha^{-1} (Mohamed Yasseen et al. 1996). *Opuntia* species have good fodder palatability (Fuentes-Rodriguez 1997) and a higher crude protein than wheat straw (Ben Salem et al. 1996). High water content in the pads helps to fulfill water requirements of animals during drought periods. Finally, these species can reduce erosion, help in land rehabilitation and be used as a refuge for wild animals.

11.5.3 Use of Crop Wild Relatives

Crop wild relatives include the ancestors and those closely related to wild species of domesticated crops. These have been exploited in plant breeding for the expansion of genetic variability in cultivated species and the improvement of various traits including drought tolerance. The Russian plant geneticist Vavilov first called attention to the potential of crop relatives as a source of novel trait variation for crop improvement (Vavilov 1926, 1949). Significant practical results were obtained in the 1970s when traits from wild species were introgressed within cultivated crops (Meilleur and Hodgkin 2004; Plucknett et al. 1987). Incorporation of genetic variability from distantly-related species to cultivated crops through interspecific or intergeneric crosses is, however, difficult and sometimes requires non-conventional methods such as embryo rescue and protoplast fusion. Interspecific and intergeneric crosses lead to complex segregation of genes and disjunction of chromosomes. Introgression from wild species is associated with linkage drag, i.e. the introgression of undesirable traits along with beneficial ones. Significant advances in the field of molecular biology, such as gene mapping, have increased the efficiency for the exploitation of wild genetic diversity (Tanksley and McCouch 1997) and reverse genetics have uncovered various drought-tolerant genes in crop species.

Some successful introgressions have been obtained in different crops. In India a drought and heat tolerant variety of chickpea (BG1103) has been released after introgression from *Cicer reticulatum* Ladiz. The variety showed superior yield and pod filling under drought stress (Hajjar and Hodgkin 2007). *Oryza longistaminata* Chev. Roehr was exploited in the Philippines for the development of drought-tolerant rice cultivars (Brar 2004). *Lycopersicum chilense* Dunal and *Lycopersicum*

Fig. 11.3 **a** *Helianthus argophyllus* Torr. *Gray*, a highly drought tolerant species in the crossing block of the College of Agriculture, University of Sargodha, Pakistan, for introgression of drought tolerance traits in cultivated sunflower, *Helianthus annuus* L.; **b** From *left* to *right*: spikes of durum wheat, *Aegilops tauschii*, synthetic hexaploid wheat (SHW) and synthetic backcross-derived line (SBL); **c** A drought tolerant synthetic hexaploid wheat accession crossed by elite bread wheat cultivar to develop drought tolerant synthetic backcross-derived lines; **d** Evaluation of synthetic backcross-derived lines (SBL) under drought conditions at the Ciudad Obregon CIMMYT station, Mexico (Photos by: M. Zaharieva)

penellii (Corr.) D'Arcy were used for the introduction of drought tolerance genes in cultivated tomato (Rick and Chetelat 1995). *Helianthus argophyllus* Torr. Gray (Fig. 11.3a) were exploited for the introduction of drought tolerance genes into cultivated sunflower.

11.5.3.1 Synthetic Hexaploid Wheat (SHW)

Bread wheat, *Triticum aestivum* L. ssp. *aestivum* (2n = 42, BBAADD) evolved from limited hybridization events between *T. turgidum* L. and *Aegilops tauschii* Coss. Evolutionary bottlenecks reduced genetic variation of the species, excluding potentially adaptive alleles (Charmet 2011). The International Maize and Wheat Improvement Centre (CIMMYT) has been strongly involved in expanding genetic diversity of wheat by re-creating synthetic wheats through intergeneric hybridization. Synthetic hexaploid wheats (SHW) have been produced by crossing the tetraploid wheat *T. turgidum* L. ssp. *durum* (2n = 28, AABB) with the diploid wheat relative *A. tauschii* (2n = 14, DD), followed by chromosome doubling of F1 hybrids through colchicine treatment (Mujeeb-Kazi et al. 1996). These primary SHW are genomically amphidiploids (2n = 42, AABBDD), combining the genomes of their parents and can act as a bridge for the introduction of specific characters and genetic diversity from their progenitors into bread wheat. SHW were involved in backcrosses with elite bread wheat cultivars to produce synthetic backcross-derived lines (SBL) with superior quality, disease and pest resistance and yield (Villareal et al. 1994; Warburton et al. 2006) (Fig. 11.3b). As the hybridization events that spontaneously formed bread wheat were limited, the genetic diversity obtained from synthetic hexaploid wheats may contain novel alleles and genes for biotic and abiotic stress tolerance not currently represented within the *T. aestivum* gene pool.

Crosses between elite wheat cultivars and synthetic wheat selected for drought tolerance under managed drought stress conditions were crossed with elite bread wheat varieties (Fig. 11.3c). The SBL obtained were evaluated in different locations (Trethowan and Mujeeb Kazi 2008) (Fig. 11.3d).

The SBL yield was 23 and 33 % higher as compared to their adapted recurrent parents and the best local check, respectively (Trethowan and Mujeeb-Kazi 2008). Yield advantage of 30 % over recurrent parents has also been reported under drought stress in advanced lines derived from crosses of SHW with Australian commercial varieties (Dreccer et al. 2008). SBL out-yielded local varieties under drought conditions in many countries (Lage and Trethowan 2008; van Ginkel and Ogbonnaya 2007). Reynolds et al. (2006) compared two SBL with their recurrent parents (Australian cvs. Cunningham and Excalibur) and standard checks (MES and Weebil-1) under non-stress and stress conditions and found that improvement in drought tolerance of SHW and SBL was due to a greater partitioning of root mass to the deeper soil profiles (60–120 cm) and increased ability to extract moisture from those depths. Four high-yielding cultivars derived from SHW (Chuanmai 38, Chuanmai 42, Chuanmai 43 and Chuanmai 47) were released in China. Chuanmai 42 had the highest average yield (>6 t ha^{-1}) among all cultivars in the Sichuan Province yield trials, out-yielding the commercial check cultivar by 22.7 % (Yang et al. 2009). Using the SHW-derived varieties as breeding parents, 12 new wheat varieties with higher grain-yield potential were recently developed (Li et al. 2014).

Cultivated emmer, *Triticum turgidum* L. ssp. *dicoccon* (Schrank) Thell. has also been used for SHW production to exploit the genetic variation for drought tolerance

present in A and B genomes (Zaharieva et al. 2010). Emmer-based synthetic backcross derived lines showed higher yield under drought-prone conditions in Mexico, Pakistan and Eastern India as compared to durum wheat (Trethowan 2014; Trethowan and Mujeeb-Kazi 2008).

11.5.3.2 Triticale and Tritordeum

Triticale (X *Triticosecale* Wittmack) is a manmade species, originally developed to combine the stress tolerance of rye and the yield potential of wheat. Hexaploid triticales (AABBRR) were developed by crossing *Triticum turgidum* ssp. *durum* with *Secale cereal* (L.) M. Bieb. Triticale is not very productive under non-stress environment (Fox et al. 1990) but produces high grain or fodder yield under drought stress when compared to durum wheat (Mühleisen et al. 2014).

Tritordeum (AABBHchHch) has been created by hybridization between durum wheat (*Triticum turgidum* ssp. *durum*) and wild barley (*Hordeum chilense* Roem. Schult.). Hundreds of tritordeum lines have been produced (Martin et al. 1996). Drought adaptation of tritordeum lines was evaluated and compared to that of wheat and triticale under different conditions. In the lowest-yielding environments, tritordeum and triticale yields were equivalent. However, under better growth conditions, tritordeum yield was lower than that of wheat and triticale (Villegas et al. 2010).

11.5.4 Chromosomal Translocation and Substitution

Translocation is the integration of non-homologous chromosomal segments when partial or complete pairing takes place not only between the homologues but also between non-homologues chromosomes. It is called alien translocation when the chromosome segment from a wild source or from species of other genera is integrated into the cultivated species chromosome. Translocations have been used to expand the genetic variability and as a source of novel traits within crop species.

Wheat rye translocations have been shown to induce drought tolerance in wheat species. Wheat-rye translocations for chromosome 1RS and 2RL have been widely exploited for wheat improvement. Chromosome 1 in rye has been considered to carry useful genes related to increased root biomass on its short arm. Therefore, short arm translocation of rye chromosome 1RS was induced in various wheat cultivars. Translocation 1RS.1BL was found useful for the production of high grain yield under drought conditions (Ehdaie et al. 2003; Hoffman 2008; Karki et al. 2014; Ko et al. 2002; Villareal et al. 1998). Translocation of rye chromosome 1RS in wheat cultivar Pavon 76 was associated with reduced plant height and increased root biomass (Ehdaie et al. 2003). However, there was no relationship between root biomass and grain yield. Pavon 1RS.1AL-1RS.1DL (R4AD) with four dosages of 1RS had the highest shoot and root biomass but the lowest yield potential under drought stress conditions (Mahepeela et al. 2014). The translocation 1RS reduced

the central metaxylem diameter, a trait that has been associated with drought tolerance in wheat (Placido et al. 2013). As a consequence, the wheat varieties developed from rye translocations are grown widely throughout the world. Schlegel (2014) documented about 1,050 varieties carrying the 1RS.1BL translocation, about 100 the 1RS.1AL translocation, and about 30 the 1R(1B) substitution.

A translocation from *Thinopyrum ponticum* (Podp.) Barkworth and D. R. Dewey (syn. *Agropyron elongatum* (Host) Beauvois), 7DL.7EL, has been shown to improve yield under drought stress conditions. The translocated line had higher seminal and lateral roots and maintained higher stomatal conductance and photosynthetic rate under drought stress (Placido et al. 2013). Transcriptomic analyses showed that the regulatory genes KNAT3 and EF2C were down-regulated in the translocated lines, resulting in enhancing lateral root development. Molnár et al. (2007) developed disomic wheat-barley (*Triticum aestivum* ssp. *aestivum* × *Hordeum vulgare* L.) substitution lines for chromosome 4H with 4D which showed higher water use efficiency under drought stress. Addition, substitution and translocation lines developed from wheat-barley hybrids were evaluated by Hoffmann et al. (2011) under stress and non-stress conditions. The authors found a large variation for traits that can be useful for the creation of new varieties with better adaptation.

11.5.5 Heterosis or Hybrid Vigor

Heterosis is the superiority of the F1 generation over the mid parent (MP) value, estimated in percentage as $\left[\left(\mathrm{F1}-\mathrm{MP}\right)/\mathrm{MP}\right]*100$, where $\mathrm{MP}=\left(\mathrm{P1}+\mathrm{P2}\right)/2$. It is called heterobeltiosis when the performance of the F1 exceeds that of the parent with higher mean value. Economic heterosis is the difference between the values of the F1 and the best commercial variety.

Hybrid breeding resulted in a remarkable increase in vigor and yield of various field crops over more than 100 years. The yield potential of a hybrid depends on the magnitude of heterosis, that in turn is influenced by the genetic distance and combining ability of the parental lines.

There are several theories regarding heterosis. The dominance theory (Davenport 1908) attributes the superiority of hybrids to the suppression of undesirable recessive alleles from one parent by dominant alleles from the other. The over-dominance theory (East 1908; Shull 1908) attributes the heterozygote advantage to the survival of many alleles that are recessive and harmful in homozygotes. A potency ratio can be calculated as (F1 − MP)/(BP − MP) where MP is the mid parent value and BP the better parent value. Potency ratios lower than 1, equal to 1 and greater than 1 correspond to partial dominance, complete dominance and over dominance, respectively.

General combining ability means the ability of a breeding line to produce superior progeny in a series of crosses, while specific combining ability is the performance of an inbred line in specific cross combination. Every year thousands of breeding lines are tested globally for general and specific combining abilities and

only few of them achieve commercial success. Heterotic groups were created by plant breeders to classify inbred lines and recurrent selection has been exploited to improve the combining ability of the breeding lines. Plant molecular techniques have been used for the constitution of heterotic groups by an estimation of genetic distance between lines.

Commercial hybrids are produced in different ways depending upon the number of inbred lines and the type of male sterility. Single-cross hybrids based on the ABR breeding lines system are used in many crops such as maize, sunflower and tomato. The A line contains a cytoplasmic male sterility source and is used as female line, the B line is used as a maintainer line of A and the R line contains fertility restoration genes in its nuclear genome and is used as pollen source for crossing A line. Double-cross hybrids are developed by crossing four genetically diverse lines.

Hybrid breeding methods were used to enhance yield potential, particularly in maize, sorghum, rice, sugar beet and sunflower. In various studies, hybrids had greater buffering capacity against yield reduction under drought stress than lines due to their heterozygous genetic background, and the magnitude of heterosis was found to increase under drought stress (Monneveux et al. 2006). Phenotypic selection for the development of drought tolerant inbred lines has, however, major effects on the performance of the hybrid. The performance of hybrids under drought cannot be predicted from only the parental inbred lines. Therefore, large numbers of crosses should be done and the resulting hybrids subjected to evaluation under multi-location trials for heterosis and secondary traits inducing drought tolerance. On the basis of performance of the hybrids under multi-environments, inbred lines can be selected for the development of commercial hybrids. Hybrids showing better performance under targeted conditions may be released for cultivation under drought-prone environments and can become the source for further cycles of selection and development of inbred lines. Continuous testing and evaluation of hybrids across broad ranges of environments brought significant genetic gains in yield under both favorable and drought stress environments, the rate of genetic gains for yield being lower under drought (Duvick et al. 2004). Significant yield gains have been, however, reported in hybrids under drought stress, attributed to an improvement of root system architecture and function that increased the capacity to extract water from the lower soil profile (Campos et al. 2006; Cooper et al. 2006; Monneveux et al. 2006).

11.5.6 Polyploidy

Polyploidy is a widely distributed phenomenon among crop plants. Many crop species are polyploids, including cultivated wheat, cotton, potato, tobacco, strawberry and cabbage. Polyploidy has played an important role in speciation, and more than 15 % of angiosperm species have originated from polyploidization. Polyploidization has induced novel features in these species allowing them to occupy new environments and increase their geographical distribution. The term polyploidy refers to the

presence of two or more copies of genomes. A polyploid species can be triploid (3×), tetraploid (4×), hexaploid (6×) or octoploid (8×). Polyploids are allopolyploid when more than two different sets of genomes are present in a single cell. Crop species such as wheat (6×), cotton (4×), tobacco (4×), peanut (4×) are allopolyploid. Bread wheat (*Triticum aestivum* L.) is an allohexaploid comprising three genomes (A, B and D) while cotton and tobacco are allotetraploids with two genomes (A and D). Autopolyploid species contain more than two copies of a single type of genome. Many crop species belong to this category such as banana (3×), potato (4×) and berseem clover (4×). Within polyploid species, 4× is the most common level of polyploidy and allopolyploidy is more common than autopolyploidy. Allopolyploid species are more adapted to diverse environments than autoploid species, the existence of diversified genome products helping to cope with environmental constraints. Autopolyploid species have some advantages over diploid species such as allelic redundancy that slows the rate of heterozygosity decay and reduces uncovering of lethal alleles. Plant breeders have induced polyploidy in crops to produce neo-polyploids.

Natural and neo-polyploid plants show better adaptability and drought tolerance. According to Ramsey (2011), neo-allohexaploid species have 70 % fitness advantage over tetraploid species. Polyploidy can increase drought tolerance in several ways. Induced polyploidy can lead to epigenetic changes within plant (brought by differential DNA methylation, histone modification or RNA interference) which alter the expression of stress adaptation related genes. These mechanisms modify the access to the genetic information by changing the state of chromatin and influencing gene expression. Allario et al. (2013) noted that induced tetraploidy modified the expression of CsNCED1, a regulatory gene of ABA biosynthesis and that neo-tetraploid species had delayed stomatal closure and growth maintenance, compared to diploid plants. Allopolyploidy species are also able to produce diversified allelic products helping them to increase their phenotypic plasticity (Adams et al. 2003). Finally, re-synthesizing the polyploid species using the original donor parental or wild species can also increase the genetic diversity within the species. Synthetic species have been successfully created in various crops including peanut, wheat and tobacco, and exploited for the improvement of drought tolerance (Leal-Bertioli et al. 2012; Lim et al. 2006; Trethowan 2014).

11.6 Breeding Schemes for Drought Tolerance

For the genetic improvement of crop species, plant breeders are generally more interested in the utilization of intra-specific variation, easily exploitable due to the absence of genetic barriers. Intra-specific crosses follow Mendelian segregation and breeder practices selection during F2 and later generations to identify promising plant progenies and pure lines. Various breeding methods such as mass selection, pure line and recurrent selection methods can be used, under stress or non-stress conditions.

11.6.1 Mass Selection

Mass selection, the simplest and least expensive selection procedure, is used to improve the overall population by positive or negative mass selection. Seeds are collected from desirable appearing individuals in the population, and the next generation is sown from the stock of mixed seed. This procedure, sometimes referred to as phenotypic selection, is based on how each individual looks. Mass selection has been used widely to improve landraces and some forage species. It is practiced within populations for traits with high narrow-sense heritability. A major inconvenience of mass selection is the large influence that the environment has on the development, phenotype and performance of single plants. Conversely, this can be an advantage in varieties to be selected for local performance.

11.6.2 Pure Line Selection

Pure-line selection generally involves the selection of superior-appearing plants from a genetically variable population, the evaluation of progenies of the individual plant selections by simple observation and further through extensive trials. Any progeny superior to an existing variety is then released as a new *pure-line* variety. The success of this method, which provided some superior pure-line varieties which are still represented among commercial varieties, depends on the existence of genetically-variable landraces. The method is still used in species that have not yet been heavily selected.

11.6.3 Hybridization

During the twentieth century, planned hybridization between carefully selected parents became dominant in the breeding of self-pollinated species. The objective of hybridization is to combine desirable genes found in two or more different varieties to produce pure-breeding progeny superior in many respects to the parental types. New allelic combinations arise due to recombination and segregation of traits. Superior plants are selected in F2 population under drought or non-stress conditions. Seeds of these selected plants are used to establish plant progenies. These progenies are grown to screen superior progenies and superior plants within superior progenies for the establishment of pure lines. Limitations of this scheme are a limited number of recombinants produced in the first three generation (F1–F3) and a low probability of changing genotypes in further generations due to rapid achievement of homozygosity (87.5 % in the F4 generation).

11.6.4 Recurrent Selection

In the recurrent selection scheme, visually selected individual plants out of the base population undergo progeny testing and those selected on the basis of the progeny test data are crossed with each other in every possible way to produce seeds to form the new base population. The intermating of F2 plants restores heterozygosity, creates new genetic variation in each population, produces new recombinants and maximizes the favorable alleles in single genotypes. These populations have been successful to break gene linkage and introduce novel recombination. The process is repeated several times and new genetic recombinations are created in each cycle of selection. Recurrent selection has been used in various crops for the improvement of yield and drought tolerance (Chapman and Edmeades 1999; Monneveux et al. 2006). Selection gains are noted after every cycle and there is no evidence for reduction in the genetic variability after each cycle of selection.

11.7 Participatory Plant Breeding

Participatory plant breeding (PPB) is the development of crop varieties through collaboration between researchers, farmers, processors and consumers. In PPB farmers define the research priorities and breeding objectives depending upon their own or local needs and are involved in the selection of segregating materials. They learn from the interaction of researchers and end users. Participatory varietal development (PVD) is a component of PPB. Selection is carried out in target environments and farmers select breeding lines that are more suited to their needs and well adapted to their conditions. The selection is practiced for specific adaptation since it is carried out within a specific environment rather than over environments. Varietal development is cheaper and more rapid in PVD than in conventional plant breeding. Moreover, PVD represents a mean of increasing genetic diversity and variability at the farm level (Atlin et al. 2001). PVD often facilitates access to improved seeds in the case of poor extension services or absence of large scale public or private seed multiplication system, which is often the case in developing countries (in Pakistan, 210 private seed companies produce only 15 % of the total improved seed demand).

National institutions conducting breeding formal (conventional) plant breeding (FPB) do not have enough resources to breed varieties that are suitable to many micro-climatic conditions or target environments. Varieties are often developed under resource-rich environments for further cultivation on a large scale and show high yield gaps (representing 30–60 % of yield potential) when cultivated in farm fields. PPB has been suggested as a suitable substitute of FPB for the development of crop varieties for poor farmers or low input environment (Bänziger and Cooper 2001). PPB is also effective to develop crop varieties for heterogeneous environments. Ceccarelli et al. (2007) noted that PPB was more effective than FPB for

handling genotype x environment interactions in drought prone regions where erratic distribution of rainfall combined with temperature stresses leads to high variation in performance over years. The same authors identified barley lines with good specific adaptation through selection for stress tolerance (low rainfall and high temperature), utilization of local adapted germplasm as parental material, use of field plot techniques to control environmental noises and involvement of farmers in the selection processes. The utility of PPB was identified as more targeted and easier to manage. Ceccarelli et al. (2001) proposed a decentralized plant breeding combining FPB and PPB. In this scheme, PPB segregating material is generated by the plant breeders in FPB programs. The material is divided among farmers of targeted environments, with each target environment being represented by a single farmer. These farmers (level-1 farmers) make the selection and then distribute the selected material to 5–10 neighboring farmers (level-2 farmers). Level-2 farmers handle 10–20 lines per year. Plant material is grown in replicated yield trials, and incomplete block design is used for evaluation. Selected plant material is transferred to 5–10 level-3 neighboring farmers who test the plant material in large plots. The specific adaptation and seed multiplication of superior bulks begins after 5 years of plant material handling. These superior bulks are also subjected to single seed descent or pure line selection methods for increasing the purity of the bulk populations.

11.8 Biotechnology Techniques to Improve Drought Tolerance Breeding Schemes

11.8.1 Doubled Haploids

The term *haploidy* is used for the gametic chromosome number and is denoted by *n*. Haploid chromosome number equals monoploid (n = x) in diploid species while in polyploid species, haploidy is called polyhaploid. In tetraploid species, gametic chromosome number is dihaploid (n = 2x). In comparison with the diploid plants, regenerated haploid plants have been shown to be weak and completely sterile (Forster and Thomas 2005). Haplodiploidization permits obtaining homozygous cells or plants issued from in vitro or in vivo methods, and homozygous transgenes from haploid embryos submitted to particle bombardment (Germanà 2011a). It is used for the rapid induction of homozygosity and the development of pure lines in self-pollinated crops, and inbred lines for the development of hybrids (Germanà 2011a, b). Doubled haploidy allows avoiding the 6–7 generations required for the induction of the homozygosity in crop plants. Selection for quantitative traits is highly effective with homozygous plants obtained through doubled haploids (Germanà 2011a). Doubled haploids increase the selection efficiency in recurrent selection when groups of selected plants are subjected to the doubled haploid induction for the establishment of a homozygous base population. Doubled haploid populations have also been utilized to develop mapping populations used in marker

assisted selection, and represent a good substitute to the recombinant inbred lines (RIL) (Germanà 2011b; Weber 2014). Finally, regeneration of dihaploid plants is convenient in autotetraploid species where induction of homozygosity is slow (Weber 2014). Gene action is complex due to the presence, for a single loci having two contrasting alleles, of four genotypes, i.e. quadriplex (AAAA, having all dominant alleles), triplex (AAAa, having three dominant and one recessive allele for single loci), duplex (AAaa, two dominant and recessive alleles) and nulliplex (aaaa, all recessive alleles) (Comai 2005; Parisod et al. 2010). In contrast, diploid species have only three genotypes with a simple segregation of alleles (AA, Aa and aa). The dihaploid regenerated plants are more fertile and can be handled as diploid species. The frequency of desirable haploid gametes is always greater than the frequency of desirable diploid embryos. Therefore, doubled haploidy is known to decrease the frequency of undesirable genotypes as desirable gametes are directly selected and regenerated to a complete plantlet (Comai 2014).

The most common method for the induction of haploidy is the in vitro regeneration of immature anther cells and the subsequent doubling of chromosomes. However, this method is genotype specific and not applicable to recalcitrant species which are not responsive to the tissue culture media for regeneration through callus culture.

In tissue culture responsive species, a large proportion of doubled haploid plants can be regenerated through microspore culture since chromosome doubling can be done earlier during in vitro culture. The system of development of double haploid through micro-spore cultures has been successfully developed in many important cultivated species such as wheat, rice, rapeseed (canola), tobacco and barley (Machii et al. 1998; Roly et al. 2014; Serrat et al. 2014; Smith 2013; Takahira et al. 2011). In vitro regeneration and screening of microspores of various cereals species is well documented (Smith 2013). Microspores culture has been used in diverse species but new variations can appear due to somaclonal variations in the callus culture (Germanà 2011a, b).

Other methods include wide crosses in which chromosomes from one parental species are rapidly eliminated at latter stages. At CIMMYT, haploid inducer maize lines have been developed for the induction of haploidy in female or male lines (Prasanna et al. 2012). The in vivo method for the induction of haploidy through haploid inducer lines is becoming popular among maize breeders (Prasanna et al. 2012). There are two modes of haploidy induction in maize, giving maternal or paternal haploids. In maternal haploids, the genome is exclusively donated by the female line while the male line induces the haploidy. This is the opposite in the paternal haploids. Haploid induction in maize inducer lines is under control of quantitative trait loci and several small loci are dispersed in the entire genome of maize. Haploid induction rate through these lines is enough for its routine use in plant breeding (Strigens et al. 2013). In order to exploit the in vivo induction of haploids, crosses are attempted between the female donor and male inducer lines. Haploid seeds are identified by the purple aleurone and scutellum due to expression of the dominant marker R1-nj in the kernels (Prasanna et al. 2012). Selected seeds are germinated and shoots tips are treated with colchicine for doubling of the chro-

mosome. Doubled haploid plants are subjected to karyotypic analysis for confirmation. This procedure has facilitated the rapid production of inbred lines and populations for mapping the QTL associated with traits related with drought tolerance (Strigens et al. 2013).

In barley, wide hybridization is exploited for the production of haploid plants (Comai 2014). Chromosomes of wild species (*Hordeum bulbosum*) are eliminated in the subsequent production of haploid plants. This system of haploid production was found to be genotype-independent and has been exploited extensively in barley breeding. More than 60 barley cultivars have been developed through this system. Wide hybridization is also practiced in other cereals such as wheat for the production of haploid plants. Maize pollen has been used to pollinate the wheat spikes for the development of haploids embryos (Khan and Ahmad 2011). These embryos are rescued from the seed and cultivated in vitro to obtain haploid plants. The haploid seedlings are then grown in a tissue culture media supplemented with colchicine for the production of doubled haploid plants (Sourour et al. 2012). Wide hybridization for the induction of haploid plants has been limited to the cereals.

In vitro or in vivo screening of haploids gametes under drought stress has been an effective tool to increase the frequency of homozygous drought tolerant genotypes (Ambrus et al. 2006). It has been noted that 65 % of the genes responsible for cell structure and tolerance to stresses are expressed during the gametophytic stage causing variation among gametophytes for drought tolerance. The undesirable (conferring drought susceptibility) haploid cells or gametes are eliminated from the population within a single generation as compared to field screening of the plants where heterozygous plants continue to show segregation for drought tolerance in every generation. In vitro screening has been carried out for the selection of drought tolerant microspores. Ambrus et al. (2006) screened maize microspores against the reactive oxygen species (ROS) induced by drought. It was noted that ROS species decreased the callus induction and regeneration potential. However, stress only allowed tolerant microspores to proliferate and to be regenerated into complete plantlets.

11.8.2 Embryo Rescue Technique

Wild species are a good source of drought tolerance and genetic variability (Sharma et al. 1996). However, success rates of wide crosses are low due to embryo abortion sooner or later after pollination. Embryo abortion occurs due to differences in ploidy levels, inhibition of chromosomal pairing, poor connection between chalzal cell and cytoplasm, degenerated endosperm and lack of starch availability at syngamy. In vitro embryo rescue techniques have been recommended to overcome post fertilization barriers. An early rescue (3–5 days after pollination) can be done to cultivate immature seeds, flowers or siliques (Smith 2013). Embryos may be directly dissected from the seed 10 or more days after pollination and grown on a suitable media to directly germinate into seedlings (Lulsdorf et al. 2014). Nutrients are

Table 11.4 Different protocols used for embryo rescue in interspecific crosses

Cross	Culture medium	Embryo age	Stress	Reference
Helianthus annuus × *H. argophyllus*	MS medium without hormone	10 days after pollination (DAP)	Drought tolerance	Sauca and Lazar (2011)
Hylocereus polyrhizus × *H. undatus*	Half-strength MS medium containing 680 µM glutamine, 0.55 µM NAA, 0.45 µM TDZ	5 DAP	Aridity	Cisneros and Tel-Zur (2010)
Aegilops tauschii × *Hordeum bulbosum*	MS medium	12 DAP	Chromosome elimination	Inagaki et al. (2014)

NAA naphthaleneacetic acid, *TDZ* thidiazuron

added in the media for the germination of the embryo under in vitro conditions since seeds developed after wide crossing usually have degenerated endosperms. Embryo rescue also reduces the time span for the next generation since rescued embryos are directly converted into seedlings (Lulsdorf et al. 2014).

The greater the incompatibility between species, the higher is the probability that an embryo may abort sooner after the fertilization. Various protocols have been proposed depending upon the degree of incompatibility, species and age of embryo at which rescue is to be done (Table 11.4).

Embryo rescue has been exploited to facilitate the introduction of drought tolerance genes from wild species. Embryo rescue techniques also successfully contributed to the introduction of novel genetic variability (Mujeeb-Kazi 2003). It facilitated the development of drought tolerant interspecific hybrids in sunflower (*Helianthus annuus* × *H. argophyllus*), Sauca and Lazar (2011), wheat (*Triticum durum* × *Aegilops tauschii*) Trethowan et al. (2014) and Brassica (*Eruca sativa* × *Brassica campestris*) Agnihotri et al. (1990).

11.8.3 Marker Assisted Selection for Drought Tolerance

Most of the economic traits including those related to drought tolerance are quantitative and strongly influenced by the environment. The term quantitative trait loci (QTL) applies to genome regions that control these traits. The progress of molecular genetics permits identification of regions which are associated with a quantitative trait. The establishment of linkage maps of various quantitative traits began in 1980s. Since then a large number of QTL studies have been carried out. After the development of mapping populations and identification of polymorphic marker, the linkage between the molecular markers and QTLs was established. These molecular markers can be further used for a marker-assisted selection (MAS). MAS permits acceleration of the breeding process. It has been applied in various crop species

Table 11.5 Comparative differences among various molecular markers used in plant breeding

Characteristics	Molecular markers			
	RFLP	AFLP	RAPD	SSR
PCR	Enzymatic cutting	Enzyme and PCR	PCR	PCR
Inheritance	Co-dominant	Dominant	Dominant	Co-dominant
Heritable	Highly heritable	Heritable	Non-heritable	Highly heritable
DNA quality	High	High	Low	High
Primer designing	No	No	Random	Specie specific
Expertise	High	High	Simple	Simple
Labor	Time consuming	Time consuming	Quick	Time consuming
Cost	Expensive	Expensive	Cheap	Expensive
Reliability	High	Medium	Low	High
Radioactivity	Present	Absent	Absent	Absent
Locus amplification	Single	Multiple	Multiple	Single
Utility	MAS	Diversity analysis	Diversity analysis	MAS

RFLP restriction fragment length polymorphism, *AFLP* amplified fragment length polymorphism, *RAPD* randomly amplified polymorphic DNA, *SSR* simple sequence repeat, *MAS* marker assisted selection

such as wheat, rice, cotton, oil seeds and forage species and represents an additional tool in breeding for enhancing yield under a drought environment (Venuprasad et al. 2009).

Molecular markers have been classified into protein-based markers (such as isozymes) and DNA based markers, the latter including restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR) and single nucleotide polymorphism (SNP). Within these markers there are many variants, each marker having its own advantages and disadvantages (Table 11.5).

Molecular markers have been shown to be helpful in the selection and improvement of complex quantitative traits (e.g. yield under drought) and traits that are highly laborious or cannot be measured in breeder segregating population due to their destructive nature (e.g. traits related to root architecture, water-use efficiency and osmotic adjustment). They are also helpful in the introgression of traits from wild genotypes in reducing number of backcrosses and linkage drag (introgression of undesirable genes along with gene of interest).

Microarray techniques have been widely exploited to understand the differential pattern of gene expression and identify drought-responsive genes, the expression of which increases under drought stress, and drought inducible genes which only express under drought stress. Huang et al. (2008) identified 2,000 drought stress responsive genes in *Arabidopsis thaliana* the expression of which increases several folds during stress treatment. About one third of genes were regulated by ABA and the ABA

analogue 1425. Concentration of ABA and its catabolites showed a significant increase under water stress and declined to a normal level within three hours after rewatering. Seki et al. (2002) identified that drought stress increased the expression of 277 genes, among them 22 were also induced by cold and heat.

11.8.4 Somaclonal Variation and In Vitro Selection

Occasionally, in vitro screening is used to exploit somaclonal variation. Somaclonal variations arise from spontaneous or induced mutations within calli and it has been noted that regenerated plantlets differ in tolerance to drought stress. Somaclonal variations are phenotypic variations due to genetic or epigenetic changes in cultured cells derived from somatic or gametic explants. Somaclonal variations are generally considered undesirable as they destroy the genetic homogeneity of the regenerated plants. Spontaneous variations in cells, however, provide breeders an opportunity to select novel variations within regenerated plants. Somaclonal variations can be permanent or temporary. Temporary variations are due to epigenetic or physiological changes within regenerated plant and are non-heritable. Permanent variations are due to mutations, polyploidy, endopolyploidy, polyteny, chromosomal aberrations, re-arrangement or activation of transposons.

There are several factors that influence the frequency of somaclonal variations, which occur in callus culture rather than in shoot tips and are more frequent in disorganized tissues. Plant-growth regulators influence the frequency of somaclonal variations. Cytokinins such as BA (6-benzyladenine or 6-benzylamino purine) at a concentration of 15 mg L^{-1} has shown to increase chromosome number in banana (Giménez et al. 2001) and genetic variability of rice plants regenerated from callus culture. Auxins that induce DNA methylation and 2,4-D (commonly used for the induction of callus) have been reported to induce polyploidy and endo-reduplication (Mohanty et al. 2008). Some genotypes show a higher frequency of somaclonal variations. Finally, sub-culturing and increasing the duration of culturing in cell suspension cultures enhance the frequency of somaclonal variations (Bairu et al. 2006).

Somaclones have been exploited for the improvement of both oligogenic and polygenic (such as yield, yield quality, biotic and abiotic tolerance) traits. Somaclonal variations are dominant and are expressed in R0 generation. Therefore, they are exploitable in crops in which sexual reproduction is not possible in several regions of the world, such as in sugarcane. In vitro culture provides an effective mean of screening cell cultures for somaclonal variations. PEG-induced drought has been used for in vitro selection of drought tolerant somaclones. The plants regenerated from seedlings having survived on the agar plate in the presence of PEG exhibited higher yield under drought stress and showed a diversity of drought tolerance mechanisms, depending upon the species (Table 11.6).

When breeding for tolerance to a given abiotic stress, part of the screening process can be developed using in vitro culture, in the presence of the corresponding

Table 11.6 In vitro selection of drought tolerant somaclones

Species and genotypes	Screening method	Screening criteria	Reference
Rice cv. PR 113	PEG 30, 50, 70 g L^{-1}	Regenerated plants (R1), Yield and components	Verma et al. (2013)
Wheat cv. GA 2002	Osmotic stress at −0.9 MPa	Variation in R1 for leaf rolling	Mahmood et al. (2012)
Rice cv. Pusa Basmati 1, Pant Sugandh Dhan 17, Taraori Basmati and Narendra 359	PEG 10, 20,30, 50, 70 g L^{-1}	Proline content, yield and morphological traits	Joshi et al. (2011)
Pineapple somaclones P3R5 and Dwarf vs. Red Spanish Pinar	No selection pressure	Stomatal frequency, photosynthesis and transpiration rate	Peréz et al. (2011)
Maize cv. KAT and PH01	Mannitol and PEG-6000	Callus survival, callus proliferation plant regeneration %	Matheka et al. (2008)

stress. Screening of germplasm for tolerance to osmotic stress can be carried out using various types of osmotica to reduce the water potential of the growth media. Screening in the presence of osmotica has been found to be advantageous as osmotic stress is more precisely controlled and screening results are repeatable. Large number of accessions can be screened through this method. However, results sometimes may be misleading due to the differential effect of osmotica when compared with soil water stress. In particular, it has been noted that mannitol is toxic to the plant. Low molecular weight osmotica may penetrate into the cell wall causing plasmolysis, i.e. a decrease in the volume of the protoplasm (Gopal and Iwama 2007) instead of the cytorrhysis (a decrease of the volume of protoplast and cell walls) produced by water stress in soil cultures. These undesirable effects can be overcome by using large molecular weight osmotica like polyethyleneglycol-6000 (PEG 6000). Polyethylene has been frequently used for in vitro screening of germplasm for seedling germination or growth. Seeds are directly germinated over the agar plates containing PEG.

The use of PEG for in vitro screening is advantageous as this osmoticum does not dry out over time. Water potential remains constant and explants have to adjust their potential to the constant low-water potential in the media. Therefore, in vitro culture is ideal for screening germplasm against dehydration tolerance. Osmotic adjustment mediated by various compatible osmolytes can be determined in the tissues or cells exposed to in vitro PEG-induced water stress. However, variation in the performance of accessions may arise due to a concentration of sucrose in the growth media. Sucrose not only imposes additional drought stress by reducing the osmotic potential of the media but also affects ABA mediated response.

In vitro screening has been used in various crop species for the identification of drought-tolerant germplasm (Al-Khayri and Al-Bahrany 2004; Biswas et al. 2002; Wani et al. 2010). Al-Khayri and Al-Bahrany (2004) noted that date palm cv. Barhee was more tolerant to drought stress in comparison to cv. Hilali, after in vitro screening

based on various traits such as callus growth rate, proline and water content. After in vitro comparison, Wani et al. (2010) noted that the rice variety PR116 was more tolerant than PAU-21. The cell membrane injury test is a rapid method for screening of large quantities of germplasm under in vitro or field drought stress conditions, based on the fact that stress damages the membrane of the plant cell, resulting in the leakage of electrolytes. A susceptible accession is assumed to show higher electrolyte leakage than a tolerant accession. Electrolyte leakage can be directly measured using an electric conductivity meter (Bajji et al. 2001; Blum and Ebercon 1980; Premachandra and Shimada 1987). The cell membrane injury test can be associated with selection in field conditions (Fig. 11.4).

11.8.5 Induced Mutation

The term *mutation* was coined by De Vries (1901) to denote a sudden change in the genome. The role of spontaneous mutations in creating new alleles had been well observed and documented by scientists at the end of the nineteenth century (van Harten 1998). However, it took almost 30 years (1927) to establish that mutations can be artificially induced by the use of physical and chemical mutagens (van Harten 1998). Since then, mutations have been applied for the improvement of various crop species and more than 2,200 cultivars have been released (Ahloowalia et al. 2004; Kharkwal et al. 2004). Mutants were easily created for mono or oligogenic traits such as plant architecture, maturity and disease resistance. However, improvement of polygenic traits including yield and tolerance to abiotic stresses have also been reported (Ahloowalia et al. 2004; Kharkwal et al. 2004). Induced mutagenesis was successfully exploited in vegetatively-propagated species such as sugarcane and fruit crops and seed-propagated species such as barley, chickpea, wheat and cotton. Mutation breeding was exploited not only to develop new cultivars with novel traits but also to study the genetic basis of plant traits.

Efforts have been made to screen ABA insensitive mutants, presumed to be more productive under drought stress conditions (Khalil 2014). Drought tolerant mutants have been identified in wheat (Njau et al. 2005) and sugar beet (*Beta vulgaris* L.) (Sen and Alikamanoglu 2012). In recent years, mutation breeding has regained popularity among molecular geneticists due to the advent of various molecular techniques, such as targeted induced local lesions in genome (TILLING) and utilization of AC/DC transposons for tagging genes, useful for screening mutant populations and tagging desirable genes. The TILLING technique, first developed in *Arabidopsis thaliana*, has been successfully applied to identify knockout mutations and allelic variants in a wide range of species (McCallum et al. 2000). It combines mutagenesis and subsequent screening of mutations in pooled PCR product and helps to identify non-sense and mis-sense mutations in the genes of interest. It does not require transformation and cell culture, and produces allelic series of mutations useful for the genetic analysis. The knowledge of the nucleotide sequences of genes of interest is however required for the genetic analysis.

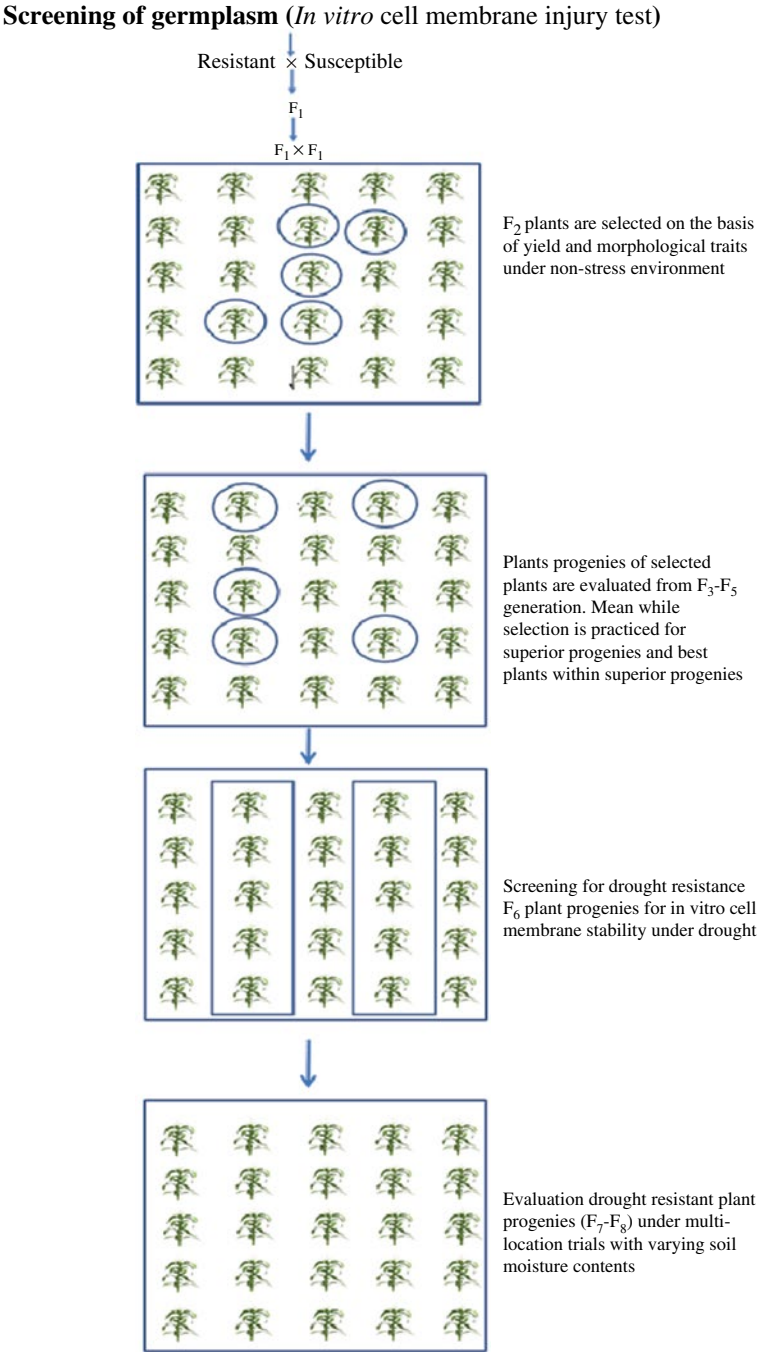

Fig. 11.4 Combination of in vitro cell membrane injury test and selection under field conditions

11.8.6 Transgenic Breeding

Transgenic plants have been successfully achieved in various crop species. Some have exhibited enhanced drought tolerance due to over-expression of transgenes under drought stress. The commercial success of these examples of transgenics is limited due to the relevance of transgenes to plant survival rather than plant productivity under drought stress (Nir et al. 2014; Zhu et al. 2014). Few commercial successes in delivering drought tolerant transgenic varieties have been reported, with the exception of MON 87460, a transgenic variety of maize over-expressing the cold shock protein B, released for cultivation in water deficit areas of the US northern Great Plains (Chang et al. 2014).

Detailed analysis of transgenes contributed to understanding functional mechanisms of drought tolerance. Nir et al. (2014) reported that the transformation of tomato plants with ATGAMT1 controlling the GA methyl transferase increased drought tolerance by reducing transpiration. Similarly, over-expression of EsWax1 increased the cuticular wax deposition on leaves and consequently reduced transpiration. However, cuticular depositions were negatively correlated with functional properties of the leaves and reduced the productivity of the plants (Zhu et al. 2014). Over-expression of the β carotene hydroxylase Chy B gene increased productivity of plants under drought stress by maintaining photosynthesis and reducing leaf necrosis under drought stress (Zhao et al. 2014). Transformation of alfalfa with GsWRKY20 increased proline and sugar content (Tang et al. 2014).

11.9 Conclusions and Prospects

Worldwide, crops are exposed to the effects of drought. Global climate change is expected to increase the occurrence and severity of drought episodes, in particular because of a higher evapotranspirative demand created by rising temperatures. Food security in the twenty-first century will increasingly depend on the release of cultivars with improved adaptation to drought conditions and yield stability. There is consequently an urgent need to improve the efficiency of breeding in developing countries, in order to increase productivity and reduce the gap between yield potential and yield in the farm fields. This requires significant advances in the understanding of mechanisms underlying resilience to abiotic and biotic stresses, together with a more efficient exploitation of genetic diversity.

Recent progress in genomics and bioinformatics are offering better opportunities to assess and enhance diversity in germplasm collections, introgress valuable traits from new sources and identify genes that control key traits. The creation of synthetic wheats and their extensive use in breeding, the development of intergeneric crosses, the monitoring and use of chromosomal translocations and substitutions represent significant practical progress, particularly for cereal crops. Significant advances have been registered in the development of in vitro selection methods and

of somaclonal variants. The manipulation of heterosis and polyploidy is offering new perspectives for improving yield potential and adaptation to abiotic stresses. Progress in the understanding of the physiological basis are expected to increase the chances to select for more efficient enzymes, thus enhancing yield potential and resilience. The level of resolution and repeatability of phenotyping methods have improved, thanks in particular to the development of remote sensing methods.

The adoption of new technologies in developing countries is, however, limited and heterogeneous, particularly in those with low or mid-level economies. This is due mainly to limited human resources and well-trained staffs, poor phenotyping infrastructure, insufficient high throughput genotyping facilities and lack of information systems or adapted analytic tools. In most of such countries, there is a need for valorizing the importance and role of agriculture and agricultural research. New incentives and funding mechanisms are needed to improve field and laboratory infrastructures, information and communication technologies and the social status of scientists.

Many of the new technologies adopted are developed in an academic context with insufficient consideration for potential applications and impact. To select plants with favorable alleles at the underlying genes and accelerate crop improvement time-scales, new technologies need to be interconnected and inserted into a global strategy. New breeding programs should ensure multidisciplinarity and, in particular, a close collaboration between genotyping and phenotyping activities. Taking full advantage of germplasm resources and genomics approaches requires an accurate and cost-effective phenotyping. Conversely, physiology activities can play an active role in the creation of new varieties only if they are fully integrated in breeding programs.

Adoption of new technologies and their better integration into breeding programs are urgently required in developing countries to overcome the bottlenecks that still limit the translation of innovations in plant science into concrete benefits for poor farmers.

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Chapter 12
Breeding Strategies for Enhanced Plant Tolerance to Heat Stress

Viola Devasirvatham, Daniel K.Y. Tan, and Richard M. Trethowan

Abstract High temperature during the reproductive period is a major limiting factor on the economic yield of crop plants. Global temperature is expected to increase 1.8–4 °C by the end of twenty-first century and impacts on plant growth and development will reduce economic yield and the quality of our food supply. Therefore understanding the effects of temperature on phenology and the physiological traits linked to tolerance is imperative if plant breeders are to develop cultivars better adapted to these hostile conditions. For example, while it is well-known that crop flowering and maturity are accelerated by high temperature, heat stress also affects photosynthesis, membrane stability, pollen fertility, fruit and/or seed yield, depending on plant species and stress intensity. Furthermore, recent advances in molecular technology have broadened the breeding strategies available to improve heat tolerance. Several crop genomes have been sequenced and a number of others are in progress, thus tools for comparing genomes and evaluating transcriptome response to abiotic stress are available or in development. Gene discovery methods and transgenic plants have helped to understand physiological traits involved with stress tolerance and to move tolerance genes between species. In addition, heat-stress tolerance is multigenic and can now be manipulated rather than only one gene at a time. These methods show potential for modifying and combining genes to meet the heat-tolerant crop needs of the future. This chapter provides an overview of the effect of high temperature stress on plant growth and development, seed quality including milling characteristics and outlines strategies to improve heat tolerance in crops.

Keywords Climate change • Gene expression • Genetic variation • Heat shock proteins • Ideotypes • Marker assisted selection • Quantitative trait

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12.1 Introduction

High-temperature stress is a serious threat to crop production worldwide and causes severe damage to plant growth, development and crop yield. Heat stress during plant growth is a complex function of temperature intensity, rate of increase in temperature and duration of high temperature and the interaction between these variables and plant growth stage and water supply (Wahid et al. 2007). Both day and night temperatures play an important role in crop productivity, particularly when high-temperature stress coincides with flowering (Wheeler et al. 2000) and evidence suggests that high temperature also limits grain quality including nutritional and milling characteristics (Ward 2007). Climate models predict that the average global temperature will increase 1.8–4 °C by the end of the twenty-first century (IPCC 2007a). The fourth assessment IPCC (2014) report highlighted the risk of additional warming above 2 °C as crop yield losses of more than 25 % and decreases in water availability are projected. The third assessment report of IPCC (2007b) highlighted increased climate variability linked to high temperature. This was confirmed by the global-scale assessment of recent observed changes by IPCC (2013) (Fig. 12.1) which showed that the frequency of heat waves has increased in large parts of Europe, Asia and Australia. Mitigation of the effects of climate change including enhancement of crop productivity and resilience under high-temperature stress are ongoing objectives in various countries.

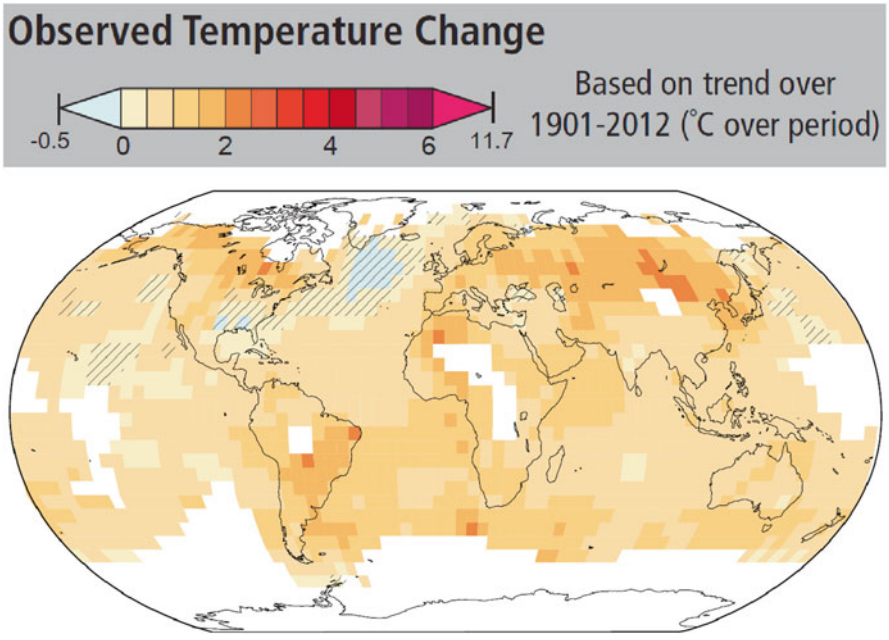

Fig. 12.1 Observed temperature change (°C) globally over the period of 1901–2012 (Reproduced from IPCC 2013)

Heat tolerance is generally defined as the ability of the plant to grow and produce economic yield under high temperature without substantial yield loss. The response to high temperature stress has been studied in various crops such as rice, wheat, maize, cotton, chickpea, groundnut, sunflower and *Brassica*. High-temperature stress tends to shorten the crop life cycle thus reducing grain yield (Craufurd and Wheeler 2009). However, earlier flowering and crop maturity can be an escape mechanism for crops grown in environments subject to terminal heat stress (Toker et al. 2007). High-temperature stress also reduces pollen viability, anthesis and grain set.

In this chapter, we present the response of various crops to high-temperature stress during the vegetative and reproductive stages by describing morphological, physiological and molecular traits that can be exploited to improve heat tolerance. Much research has been conducted to identify the sources (germplasm and genotypes) of heat tolerance in various crops. Breeding populations are available to explore the inheritance of heat tolerance in many species and these have been used to map quantitative trait loci (QTL) and identify linked markers for marker- assisted selection (MAS). This chapter explores the plant response to high temperature and the implications for stress tolerance breeding in plants.

12.2 Effect of High Temperature During Vegetative Period

High-temperature stress alters the physiological processes of the plant and the pattern of plant development. These responses may differ from one physiological stage to another. Heat stress affects seed germination, reduces seedling vigor and limits seedling establishment. This was observed and documented in grain legumes such as chickpea, lentil, soybean and cowpea (Covell et al. 1986). Coleoptile growth in maize was reduced at 40 °C and stopped completely at 45 °C (Weaich et al. 1996). A temperature increase of 1 °C was observed to shorten the number of days from sowing to heading by 4–5 days in some rice genotypes (Nakagawa et al. 2001). High temperature reduced inter-node length in sugarcane (Ebrahim et al. 1998) and relative growth rate and net assimilation rate in maize and pearl millet due to reduced leaf expansion (Ashraf and Hafeez 2004). Generally, high temperature reduces the first inter-node length resulting in seedling death or stunting (Hall 1992).

During the vegetative stage, high temperature can reduce leaf photosynthesis and carbon dioxide assimilation rates by reducing leaf area and stomatal apertures. In a controlled environment study, it was reported that maize leaf temperature greater than 30 °C affected net photosynthesis through rubisco inactivation (Crafts-Brandner and Salvucci 2002). Under high temperature (42 °C), photosynthesis was reduced in heat susceptible cotton genotypes (Sicala 45, Sicala V-2) and this was related to low membrane integrity (Cottee et al. 2010). Overall, physiological function of a range of proteins, enzymes and metabolic processes are suppressed by high temperature (Burke et al. 1988). High temperature (30/25 °C) decreased chlorophyll variable fluorescence (Fv), a measure of injury to photosynthesis, in field pea, faba

bean and common bean (McDonald and Paulsen 1997). The above research confirmed that photosynthetic activity in thylakoids was sensitive to high temperature, suggesting that Photosystem II was the most sensitive process in the plant. A heat shock (30–38 °C) for 3 h at midday for 3 days at the end of tillering in wheat reduced the rate of photosynthesis (Schapendonk et al. 2007). A similar heat shock during grain filling decreased both rate of photosynthesis and grain growth. These authors observed the rate of photosynthesis decreased by 40–70 % depending on cultivar and growth stage. To achieve improvements in a wider range of environmental conditions, a better understanding of source (photosynthesis) and sink (grain growth) under high temperature is needed.

12.3 Effect of High Temperature During Reproductive Period

Grain yield during the reproductive period is a result of total dry matter production in the pre- and post-anthesis periods. High-temperature stress during late vegetative and pre-anthesis periods can modify the crop phenology, e.g. time to opening of first flower and crop maturity. This was observed in lentil (Erskine et al. 1990), chickpea (Devasirvatham 2012), wheat (Wardlaw and Moncure 1995) and maize (Cicchino et al. 2010). Heat stress during anthesis and post-anthesis can cause flower abortion due to pollen sterility and also adversely affects stigma-style and ovary, subsequently reducing crop yield. In wheat, temperatures >30 °C during flowering caused pollen sterility (Saini and Aspinall 1982). Generally, male sterility (loss of pollen viability) due to high temperature stress pre-anthesis stress is common in wheat (Chakrabarti et al. 2011), rice (Endo et al. 2009), barley (Abiko et al. 2005), cowpea (Ahmed et al. 1992), chickpea (Devasirvatham et al. 2013), groundnut (Prasad et al. 1999), sorghum (Prasad et al. 2006) and common bean (Gross and Kigel 1994). Both male and female sterility was observed at high temperature in *Brassica* (Young et al. 2004) and tomato (Foolad 2005). However, asynchrony of male and female organs was observed, particularly in maize under temperature stress (Zinn et al. 2010). Clearly, plant responses to high temperature stress vary with species and developmental stages. In some species, such as chickpea, selection based on pollen viability can improve heat tolerance (Devasirvatham et al. 2010). Furthermore, genes responsive to high temperature in anther tissue were identified in rice (Endo et al. 2009). This approach could be integrated in crop improvement programs.

The net result of heat stress during the reproductive period is lower crop yield. In cereals, grain yield reduction was related to lower spikelet number per spike, grain number per spikelet and grain weight (Ferris et al. 1998; McMaster 1997). In wheat, high temperature tends to speed up development of the spike (Porter and Gawith 1999). However, the duration of double ridge to anthesis was reduced due to heat stress and consequently spikelet number per spike and grain number per spikelet were reduced (McMaster 1997). Similarly, the duration and rate of spikelet formation are controlled by high temperature in maize (Otegui and Melon 1997). A con-

trolled environmental study showed that heat stress during the reproductive period in sorghum delayed time to panicle emergence and flowering, which led to significantly lower grain yield (Prasad et al. 2008).

Reduction in cotton yield with high temperature stress was attributed to reduced boll size and seed number, and increased fruit shedding (Hodges et al. 1993; Pettigrew 2008). In pulses, high temperature reduced grain yield due to lower pod set, seed set and grain weight. This has been documented in mungbean (Khattak et al. 2006), groundnut (Prasad et al. 1999), chickpea (Devasirvatham 2012; Krishnamurthy et al. 2011), and field pea (Guilioni et al. 1997). *Brassica* seed yield decreased with heat stress during flowering. The reduction in *Brassica* seed yield was due to a reduction in flower number, and number and size of the seeds produced per flower (Morrison and Stewart 2002).

Heat stress generally accelerates the rate of grain filling and shortens the grain-filling duration thus hastening the physiological maturity (Dias and Lidon 2009). In chickpea, high temperature (35.7–37.5 °C) reduced grain filling duration by 4–19 days in sensitive genotypes (Devasirvatham 2012). Streck (2005) estimated that for every 1 °C rise above optimum temperature, the duration of grain filling in wheat was reduced by 3 days. However, in maize, high post-anthesis temperature stress (from 15 days after pollination to maturity) lengthened the duration of grain filling and reduced kernel growth rate (Wilhelm et al. 1999). Assimilates are transferred either from pre-anthesis stored stem reserves or from current assimilation during grain filling which can change during heat stress (Blum 1998; Palta et al. 1994). Assimilate transport from flag leaf to grain was reduced in cereals by temperatures ≥30 °C (Wardlaw 1974). Tayo and Morgan (1979) demonstrated that the number of pods and seeds per pod were regulated by the capability of *Brassica napus* to supply carbon to the inflorescence during the 3-week period following anthesis. Similarly, Morrison and Stewart (2002) found that heat stress during flowering may limit photoassimilate production and translocation to developing seeds, resulting in pods with fewer seeds of lower weight.

Generally, high-temperature stress reduces grain weight by up to 30 % in wheat depending on the genotype, timing and duration (Stone and Nicolas 1994, 1995). Rice seed weight also decreases with high temperature (Tamaki et al. 1989). Therefore, the effect of heat stress during reproduction can generally be classified into (1) reduced flower number (2) greater flower abortion due to pollen sterility or ovary abnormalities and (3) reduction in the remobilization of the photosynthates to seeds post-anthesis.

12.4 Effect of High Temperature on Seed Quality

High temperature during seed development has a strong influence on both seed quality and quantity (Zhao et al. 2008). Heat stress during grain filling increases protein content in wheat and barley (Blumenthal et al. 1991) through reduction in starch deposition (Hurkman et al. 2003), thus increasing the concentration of protein per

unit of starch (Stone and Nicolas 1998). However, heat stress during early grain filling has a greater impact on grain quality than late grain filling (Castro et al. 2007). In barley, poor malting quality is associated with high protein percentage under high temperature (Wardlaw and Wrigley 1994). Savin and Nicolas (1996) revealed that protein percentage in barley grain increased when high temperature was severe (40 °C at 6 h/day for 10 days) enough to reduce starch accumulation. This suggests that starch accumulation is more sensitive to high temperature than protein accumulation. In wheat, dough strength was reduced in response to a few days of heat shock (>32 °C) (Blumenthal et al. 1991). Insoluble protein polymers, glutenin to gliadin ratio and the starch granule size in wheat are important determinants of baking quality. Heat stress (35/20 °C) was reported to reduce the size of the starch granules in the wheat cultivar Plainsman (Balla et al. 2011). High temperatures between flowering and maturity reduced amylose content in rice and changed many of the functional properties of the flour (Zhong et al. 2005). During grain filling in rice at high temperature (36/27 °C), the number, amount and average weight of the amylase chain decreased (Ward 2007). Therefore, the modified amylase content of the starch affects the cooking quality of rice. Similarly, starch, protein and oil content of the maize kernel reduced under a high temperature regime of 33.5/25 °C exposed from 15 days after pollination until maturity (Wilhelm et al. 1999).

Isoflavone compounds (daidzein, genistein and glycetin) in soybean have potential health benefits such as bone resorption in women (Ma et al. 2007), ovarian and colon cancer cell growth inhibition (Chang et al. 2007a, b; MacDonald et al. 2005) and serum LDL cholesterol reduction (Taku et al. 2007). Temperatures >27 °C during soybean seed development were correlated with a reduction of the isoflavone compounds (Morrison et al. 2010). High temperature reduces seed size in pulses, particularly in temperature sensitive chickpea genotypes (Devasirvatham 2012).

High temperature has been associated with a decrease in sunflower oil yield (for a review, see Kalyar et al. 2014). Mean daily temperature >30 °C during the early grain-filling period (10–12 days after anthesis) reduced both the rate and duration of oil deposition in the sunflower seeds (Rondanini et al. 2006). In cotton, high temperature (30/22 °C) decreased the number of fibers per seed and seed set (Arevalo et al. 2008). Reddy et al. (1999) showed that temperatures higher than 26 °C increased short fiber in upland cotton. Therefore, heat tolerant genotypes that produce fruit and/or seed with stable nutritional quality, including dough and cooking quality are needed.

12.5 Role of Heat Stress Proteins in Plants

The expression of heat stress proteins is an important plant adaptation to high-temperature stress. The production of low and high molecular weight heat-shock proteins (HSPs) are widely reported in many crops. These proteins show tissue and organ specific expression with folding and unfolding of cellular proteins that protect functional sites from the adverse effects of high temperature (Wahid et al. 2007).

Other stress proteins reported in plants include ubiquitin, late embryogenesis abundant (LEA) proteins and pirproteins. These proteins appear in protein degradation pathways and protect against the adverse effects of oxidative stress and dehydration during heat stress (Schoffl et al. 1999). Using proteomics, Nadaud et al. (2010) determined stress proteins particularly HSPs (70, 80, 90) between 150 and 280° days after anthesis in winter wheat. The relatively heat tolerant wheat cultivars (DL 153-2 and HD 2285) showed a greater increase in HSP 18 compared with heat-susceptible cultivars (HD 2329 and WH 542) (Sharma-Natu et al. 2010). Under heat stress, ubiquitin was found in soybean (Ortiz and Cardemil 2001).

12.6 Strategies to Improve Heat Tolerance

The success of plant breeding programs is often influenced by genotype x environment (G x E) interactions and correlations among traits that improve yield and quality (Podlich et al. 1999). Generally, breeders attempt to develop widely adapted cultivars with minimum G × E influence on agronomic and grain quality characters, thereby directly or indirectly selecting for stability across the production areas (Chenu et al. 2011). Time-of-sowing field experiments and the extensive use of controlled environment facilities have provided information on high temperature tolerance. Molecular markers also play an important role in assembling the heat-tolerant crop ideotype once marker-trait associations have been established for temperature tolerance. Heat-tolerance research conducted in various crops using both controlled environments and the field is described in Table 12.1.

12.6.1 Prediction of the Effects of High Temperature Stress

It would be of considerable value to plant breeders if the changes in crop development, grain yield and quality resulting from high temperature could be predicted using meteorological data during the crop-growing period (Wrigley et al. 1994).

Table 12.1 Heat tolerant works carried out on different crops

Traits	Crops	Reference
Chlorophyll fluorescence	Wheat	Yang et al. (2002)
Spikelet fertility	Rice	Weerakoon et al. (2008)
Photosynthetic rate and membrane stability	Cotton	Cottee et al. (2010)
Pollen viability	Maize	Cicchino et al. (2010)
Canopy Temperature Depression (CTD)	Wheat	Rosyara et al. (2010)
Time of flowering and spikelet fertility	Rice	Shah et al. (2011)
Chlorophyll fluorescence	Wheat	Sharma et al. (2012)
Pollen viability	Chickpea	Devasirvatham et al. (2013)
Grain weight and oil yield	Sunflower	Kalyar et al. (2014)

In addition, there is a need to collect experimental site data such as soil type, soil water content and the cropping system to characterize the screening environment (Garrity 1984) and origin of the germplasm (Berger et al. 2006). The environmental variables associated with G × E during different phenological stages of bread wheat were used at the International Maize and Wheat Improvement Center (CIMMYT) to predict the impacts of temperature on yield (Reynolds et al. 2002). They concluded that maximum temperature 30–32.5 °C, during the later stages of grain filling were associated with lower yield in Mexico. At this site, the spike primordia growth stage was the most sensitive to environmental factors contributing to G × E. Differential adaptation of bread wheat to various heat-stressed environments around the world was analyzed by cumulative cluster analysis of locations and genotypes (Lillemo et al. 2005). The grouping pattern of wheat-growing environments could mainly be explained by the temperature at different growth stages. A clear distinction was observed between sites with heat stress and those with more specific terminal heat stress. Similarly, the analysis of G x E on chickpea heat-tolerance screening was calculated using data collected from field experiments conducted at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and reduced grain filling period was linked with lower grain yield (Devasirvatham et al. 2012). In Australia, major wheat-growing areas were used to standardize phenotyping protocols (Rebetzke et al. 2012). Desirable measurements for phenotyping yield and other traits in drought- and heat-stressed environments were identified and a national testing facility was established across three key and contrasting environments. More than 1,000 wheat genotypes were phenotyped for heat tolerance using chlorophyll a fluorescence and 41 contrasting genotypes with inherent photochemical efficiency were identified for future studies. Ultimately, the complex quantitative traits observed in most heat-stress studies should be dissected into simpler genetic factors and high-density genotyping and high-throughput phenotyping will facilitate this process (Sharma et al. 2012).

12.6.2 Genetic Variation and Breeding for High Temperature Tolerance

Field experiments with different sowing dates can be used to study yield phenological and physiological responses of genotypes to the timing and degree of high-temperature stress. Controlled environment facilities are also useful for increasing the precision of genotype responses to high temperature. Genetic variation in relation to heat tolerance in cultivated and wild wheat has been reported by many researchers (Al-Karaki 2012; Waines 1994; Wardlaw 1994; Yang et al. 2002). Similarly, genetic variation for heat tolerance in rice (Nakagawa et al. 2001), cowpea (Ehlers and Hall 1998), cotton (Azhar et al. 2009), lentil (Erskine et al. 1990, 1994), mungbean (Khattak et al. 2006) and chickpea (Krishnamurthy et al. 2011) has also been reported. Phenotypic screening based on different methods such as

canopy temperature depression (CTD), electrolyte leakage (an index of membrane stability), variable chlorophyll fluorescence or photosynthesis impairment (Fv), and pollen fertility are useful traits and correlated with yield. Considerable variation for physiological traits such as CTD, membrane stability and injury to photosynthesis has been observed in cereals, pulses and cotton under high temperature. Genetic variation for CTD under high temperature was reported in wheat (Rosyara et al. 2010) and chickpea (Devasirvatham 2012) and was correlated with yield. In both crops, heat-tolerant genotypes showed higher CTD under stress. Genetic variability in photosynthetic rate under high temperature has been demonstrated in peas in controlled conditions (McDonald and Paulsen 1997). In the field and in controlled environments, genotypic differences were observed in cotton for photosynthetic rate and membrane stability (Cottee et al. 2010). At the seedling stage, significant variation among genotypes for membrane stability was observed in chickpea, lentil and faba bean (Ibrahim 2011), highlighting the efficiency of membrane stability in selection for heat tolerance at the early stages of crop growth in food legumes. Genetic variability in pollen fertility (% pollen germination) was observed under high temperature in chickpea and was correlated with percentage pod set (Devasirvatham et al. 2013). Similarly, genotypic differences in spikelet fertility were found in rice under high temperature which subsequently reduced grain yield (Weerakoon et al. 2008).

Secondary traits such as stay-green in wheat (Reynolds et al. 1998) and delayed leaf senescence (DLS) in cowpea (Gwathmey et al. 1992) confer yield advantages under heat stress. Genetic variation for stay-green was observed in wheat under heat stress (Sharma et al. 2005) and linked to yield. Delayed leaf senescence in cowpea produced greater yield with resistance to premature death under stress (Gwathmey et al. 1992). Ismail et al. (2000) developed F_6 and F_7 recombinant-inbred lines varying for the DLS trait under heat stress and found that DLS greatly increased plant survival and individual seed size in cowpea under heat stress.

Mutants are potentially valuable sources of genetic variation obtained from either spontaneous or induced mutation that can be exploited in plant breeding (Jiang and Ramachandran 2010). Genetic mutation can also be used to investigate gene function. The relative thermo-tolerance of ten mutants and the wheat cv. Guardian were tested at 38 °C for 6 h in every 24 h between seedling and anthesis. Net photosynthetic rate (P_{max}) and chlorophyll content were compared in stressed and non-stressed conditions. The P_{max} of Guardian was depressed at anthesis but one of the mutant lines remained unaffected by heat stress at second node and ear emergence (Mullarkey and Jones 1999). Recent work with rice mutants showed promising results. Characterization of ethyl methane sulphonate (EMS) induced mutants of N22 for water stress and heat tolerance was reported by Panigrahy et al. (2011). Poli et al. (2013) studied the previously isolated mutant N22-H-dgl219 (NH219) and reported the accumulation of reactive oxygen species in the leaf under 40 °C heat conditions. The mutant was characterized for several traits in the field under ambient (38 °C) and heat stress (44 °C) conditions by raising temperature artificially from flowering stage until maturity.

Yield traits were mapped in 70 F_2 segregants of IR64×NH219 and 36 F_2 segregants of its reciprocal cross. The mutant NH219 showed more tolerance to heat stress than N22. Single-marker analysis showed significant association of RM1089 with number of tillers and yield per plant, RM423 with leaf senescence, RM584 with leaf width and RM229 with yield per plant (Poli et al. 2013).

Heat-tolerant and heat-susceptible genotypes have been crossed in different crops and the populations screened under heat stress. A double haploid population of spring wheat was screened under heat stress and a few lines yielding more than their parents under stress were identified. These lines showed variation in grain yield, thousand-grain weight, grain filling duration and canopy temperature (Tiwari et al. 2012). In cowpea, reciprocal crosses were made between a heat-tolerant (reduced leaf electrolyte leakage under heat stress) and a heat-sensitive line and random inbred lines were produced. Selected F_8 lines were evaluated under high temperature (40 °C) in the field. A strong negative genetic correlation was observed between leaf electrolyte leakage and pod set in a hot (40 °C) long-day environment but not in a short-day glasshouse (36 °C) or under moderate temperature (35.5 °C) under long-days in the field. These results indicate that several breeding cycles are needed to identify lines with leaf electrolyte leakage as low as the parents (Thiaw and Hall 2004). Two heat- tolerant and two heat-sensitive chickpea genotypes were crossed and the populations (F_1 and F_2) and parents were screened in the field under high temperature. The heritability of days-to-first flowering of these crosses was higher than other traits such as total pod number, filled pod number, seed number per plant and grain yield indicating that the timing of flowering in chickpea under high temperature is under genetic control and likely to have polygenic inheritance (Devasirvatham 2012). The adaptation of chickpea to high temperature may also be improved using more exotic parents to combine allelic diversity for flowering time, filled pod numbers, seed number per plant and grain yield. Shonnard and Gepts (1994) suggested that a single gene controlling growth habit was linked to heat tolerance during flower-bud formation in common bean. They also concluded that additive gene effects were involved in bud abortion at high temperature. Rainey and Griffiths (2005) also identified an association between a flower-abscission gene and genes controlling pod number in beans under high temperature.

12.6.3 Generating High Temperature Tolerant Transgenic Plants

High-temperature tolerance in transgenic plants has been achieved either by overexpressing heat shock protein (HSP) genes or by altering levels of heat-shock factors that regulate expression of heat-shock and non-heat-shock proteins (Grover et al. 2013). HSP genes, metabolic proteins and transcription factor genes have shown promising results under high-temperature tolerance in crops. Selected examples in increased high- temperature tolerance associated with transgenes are discussed in Table 12.2. However, most of these experiments were conducted in the laboratory and field validation remains to be done.

12.6.4 Use of Molecular Markers

Molecular markers are particularly useful for gene or QTL pyramiding. In tomato, RAPD (random amplified polymorphism) markers were used to identify yield traits under heat stress (Lin et al. 2006). Using F_7 RILs (recombinant inbred lines) derived from heat-tolerant and heat-sensitive parents, they identified 14 RAPD markers that were associated with heat tolerance. Four of these markers contributed to high fruit number, high fruit weight and high yield under heat stress conditions (Lin et al. 2006). In rice, 2 QTLs affecting pollen fertility under heat stress were identified using RILs and localized on chromosome 4 (Ying-hui et al. 2011). In maize, 11 QTLs controlling pollen germination and pollen tube growth under heat stress were identified using restricted fragment length polymorphism (RFLP) markers (Frova and Sari-Gorla 1994).

While QTL studies in crop species for stress adaptation and disease resistance are many, they are generally based on bi-parental populations and therefore sample limited genetic variation. Association analysis (AA) is now used routinely as an alternative to traditional bi-parental linkage mapping to identify genotype-phenotype associations across much wider ranges of materials. This technique of molecular marker-trait association estimation is based on linkage disequilibrium (LD) (Al-Maskri et al. 2012). Meaningful AA depends on LD, which assumes non-random association of alleles at different loci. In other words, there is an assumption that many generations of meiosis have removed associations between QTL and any markers not tightly linked to the QTL. A genetic marker close to and in LD with a targeted trait will show significant allele frequencies in the genome (Painter et al. 2011). These genome-wide scanning approaches allow researchers to search the genome for genetic variation in the targeted traits (Alonso-Blanco et al. 2009); in particular, complex traits such as drought, high temperature and salinity tolerance. Effective AA is based on LD which tends to be maintained between loci (Neumann et al. 2011). However, there is the possibility of false positive correlation between a marker and a trait resulting in bias and meaningless associations (Neumann et al. 2011). Hence, separating LD from physical linkage population structure is important and its estimation is a prerequisite in association analysis (Crossa et al. 2007). These methods were used in AA in chickpea for identifying genomic regions linked to drought tolerance (Kebede 2012; Nayak 2010).

Generally, these AA studies have been reported on experiments grown under favorable conditions. The large AA study on wheat by Crossa et al. (2007) identified diversity arrays technology (DArT) marker associations with resistance to stem rust, leaf rust, yellow rust and powdery mildew and grain yield. Crossa et al. (2007) reported many chromosome regions associated with disease resistance and grain yield, and some of these regions did not contain previously identified genes. An exception is a durum wheat study that targeted drought adaptive traits such as plant height, heading date, peduncle length, grain yield, kernels m^{-2}, thousand-kernel weight and test weight using AA (Maccaferri et al. 2011). These authors concluded that major loci for phenology were responsible for the control of drought response. AA was conducted to identify chromosomal regions and linked markers that con-

Table 12.2 High temperature stress tolerance in crops by transgenic research

Protein	Source species	Host species	Promoter	Comments	Reference
HSP genes					
HSP16.9	*Zea mays*	*Nicotiana tabacum*	CaMV35S	Overexpression in increased seed germination rate, root length and anti-oxidant enzyme activity during heat stress (40 °C; 9 h)	Sun et al. (2012)
HSP22	*Z. mays*	*Arabidopsis thaliana*	CaMV35S	Overexpression resulted in increased heat stress tolerance and altered expression of HSP genes (42 °C; 30 min)	Rhoads et al. (2005)
HSP26	*Oryza sativa*	*Festuca arundinacea*	CaMV35S	Reduction of electrolyte leakage and accumulation of thiobarbituric acid-reactive substances and higher PSII activity on exposure to heat stress (42 °C; 24 h)	Kim et al. (2012)
HSP70	*O. sativa*	*O. sativa*	CaMV35S	Overexpression manifested enhanced tolerance to heat stress (48 °C; 15 min)	Qi et al. (2011)
HSP100	*O. sativa*	*N. tabacum*	CaMV35S	Transgenics survived HT stress (50 °C; 50 min) relatively better than non-transgenic control plants.	Chang et al. (2007b)
HSFA1	*Glycine max*	*G. max*	CaMV35S	Enhanced heat stress tolerance through activation of GmHSP70 in transgenics	Zhu et al. (2006)
Metabolic proteins					
EFTu1 (elongation factor)	*Z. mays*	*Triticum aestivum*	Maize Ubi1	Transgenics had reduced thermal aggregation of leaf proteins, lesser injury to photosynthetic membranes, enhanced rate of CO2 fixation and improved tolerance to HT stress (45 °C; 18 h)	Fu et al. (2008)
CYP (cyclophilin)	*Cajanus cajan*	*A. thaliana*	CaMV35S	Transgenics showed high survival rates, biomass, better root growth and chlorophyll content. Increased tolerance to extreme temperatures (4 °C; 7 days, 37 °C; 90 min: 42 °C; 2 h)	Sekhar et al. (2010)
CEST (chloroplast protein-enhancing stress tolerance)	*O. sativa*	*A. thaliana*	CaMV35S	Overexpression resulted in better photosynthetic activity and reduced photo-oxidative damage leading to improved tolerance to HT stress (32 °C)	Yokotani et al. (2011)
Transcription factor gene					
TaHsfA2d	*T. aestivum*	*A. thaliana*		Overexpression of transgenic *Arabidopsis* plants shower tolerance to high temperature	Chauhan et al. (2013)
EsDREB2B	*Eremosparton songoricum*	*N. tabacum*		Heterologous expression of this gene in tobacco showed improved tolerance to multiple abiotic stresses	Li et al. (2014)

tribute to heat tolerance in chickpea. In this study, 359 DArT markers were assessed of which 107 markers were linked with 11 agronomic traits expressed under heat-stressed and non-stressed conditions. The phenology-marker associations under heat stress were located on chromosome 2. This study also identified new genomic regions for days to first flower, days to 50 % flowering, days to first pod, total pod number, filled pod number, biomass and plant width which were not linked to previously reported QTL (Devasirvatham 2012).

12.7 A Strategy for Developing Heat Tolerant Wheat Cultivars for Northwestern NSW, Australia

This section attempts to develop a genetic ideotype for heat tolerance in wheat for a specific region; northwestern New South Wales (NSW), using the knowledge cleaned from various crops and reported earlier in this chapter. Periods of high temperature (i.e. heat waves) have been, and continue to be, a major threat to wheat yield and grain quality throughout much of the Australian wheat belt. Present projections of climate change in Australia indicate that heat waves will become more frequent and more intense in the coming decades (Alexandra 2012). It is therefore urgent that wheat germplasm with superior high temperature tolerance is identified which can be introduced into commercial wheat breeding programs.

The northwestern NSW region (e.g. Narrabri) is one of the most important grain-production regions in Australia. The growing conditions at Narrabri (30.34°S, 149.76°E; 212 m elevation), where the University of Sydney has a major grain research station, are characteristic of the Australian subtropics. The climate is characterized by hot summers and mild winters with a summer-dominant rainfall pattern (Nicholls et al. 1997). The average annual rainfall at Narrabri is 660 mm, and the average annual maximum and minimum temperatures are 26.5 °C and 11.7 °C (Bureau of Meteorology 2011), respectively. The experimental soil is a cracking montmorillonitic clay soil classified as Grey Vertosol (Isbell 2002) with a high plant-available water capacity (>200 mm). Success in the production of wheat depends largely on the amount of summer rainfall and stored soil water before sowing. Temperature shock (that is periods of high maximum temperatures for 2–3 days at a time) tends to occur from flowering onwards.

Wheat is sensitive to high temperature particularly during flowering and post-anthesis and temperature stress causes lower grain yield. The most important trait influencing yield under heat stress is flowering time. Early flowering genotypes with a shorter growing season, more rapid grain-filling and deeper roots that are able to access deep soil moisture are potential targets for this environment. A suitable ideotype for this environment and future, warmer scenarios would also include traits that increase the efficiency of photosynthesis, increase plant growth rate, improve transpiration rates to match the high evaporative demand and maintain yield and seed weight under hot conditions (Reynolds et al. 2007). However, con-

servative traits such as transpiration efficiency (determined using carbon isotope discrimination) should be avoided as farmers make most of their income in the better years, and genotypes that are more responsive to available moisture will contribute more to farmer profitability than those that maintain a marginally-better yield under stress.

Other physiological traits correlated with yield under heat stress in wheat are canopy temperature and water-soluble carbohydrates (WSC). Higher canopy temperature depression pre-flowering tends to be associated with reduced plant height, greater harvest index and grain yield with no appreciable effect on above-ground biomass, whereas cooler canopies post-flowering tends to be associated with higher above-ground biomass, greater grain number and increased grain yield (Rebetzke et al. 2013). Cooler canopies are also associated with increased rooting depth where water is limited but present at depth (Lopes and Reynolds 2010). Clearly, cooler canopies would be a target for selection in this environment as the crop generally develops on stored soil moisture.

Pre-anthesis accumulated water soluble carbohydrates (WSC) in wheat provide an alternative source of assimilates to current photosynthesis for grain filling. At higher temperatures, a reduction in floret fertility is associated with a decrease in soluble sugars and this response is exacerbated in genotypes low in WSC. Four recombinant inbred lines of wheat (SB003, SB165, SB062 and SB165 from the Seri/Babax population) with contrasting WSC were grown at the University of Queensland, Gatton at high temperature (28/14 °C). High WSC lines (SB062, SB165) had more grains per spike associated with more florets per spike. The number of fertile florets was associated with spike biomass at booting and, by extension, with glucose concentration; both were higher in high WSC lines. At higher temperature, the intrinsic rate of floret development before booting was lower in high WSC lines (Dreccer et al. 2014). However, WSC tends to be more useful trait in southern NSW as a predictor of yield under water stress than in northwestern NSW (Ovenden et al. 2012; van Herwaarden et al. 2003). For this reason WSC would not be considered as critical to defining an ideotype adapted to high temperature in northwestern NSW.

Most of today's wheat varieties have, in the distant past, been derived from landraces (Trethowan and Mujeeb-Kazi 2008). However, considerable unexploited variability for heat tolerance still exists among landraces (Reynolds et al. 2007). Furthermore, heat-tolerant landraces tend to have higher leaf chlorophyll content and higher stomatal conductance (Hede et al. 1999). These materials could be used in breeding programs to improve heat tolerance in wheat in northwestern NSW.

A heat tolerant ideotype for northwestern NSW is described in Fig. 12.2. It would be early flowering with an inherently faster grain filling rate and would be characterized by: larger seed and grain weight that is maintained under heat stress, better pollen viability under high temperature, deeper roots, cooler canopies, lower transpiration efficiency and resistance to stem and leaf rust; two key diseases that tend to proliferate at higher temperatures. The ideotype would also have strong dough as cultivars with high grain protein and strong dough meet the regional market requirements. As temperatures increase dough strength tends to decrease glute-

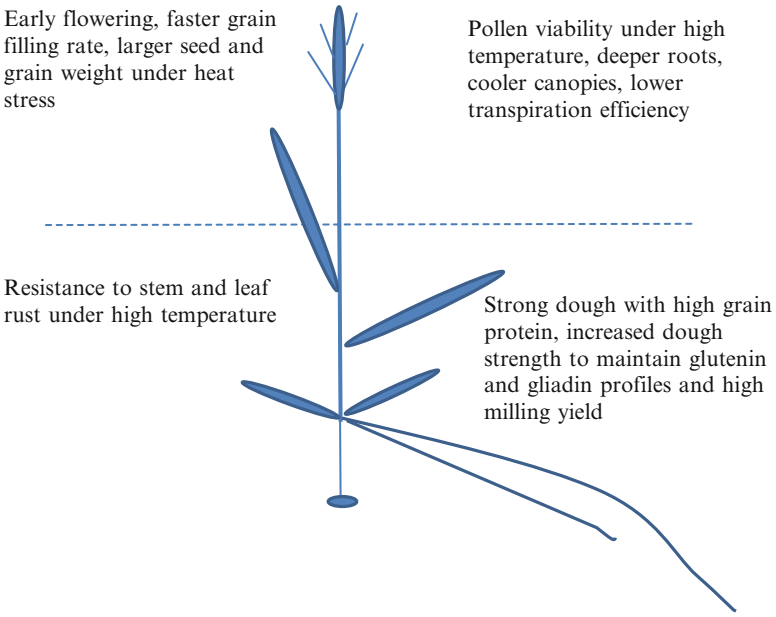

Fig. 12.2 A theoretical heat tolerant wheat ideotype for northwestern NSW

nin and gliadin profiles that increase dough strength should be selected. Maintenance of seed weight under stress and reduced screenings (the presence of small shriveled seed) should be minimized to maintain high milling yield.

The main aim of any breeding program in this region is to identify wheat germplasm with superior tolerance to short episodes (e.g. 1–3 days) of high temperature (>35 °C) using a combination of field and controlled environment screening. Specific objectives would be: (a) identification of genetic variability for heat tolerance in wheat including the traits in Fig. 12.2, (b) determination of the physiological basis of stress tolerance among the most heat-tolerant materials in both the field and under controlled conditions. In this instance controlled screening would be used to confirm trait expression (evaluated first in the field) and to maximize genetic variance for fine mapping; (c) elucidate the probable genetic control of heat tolerance using traditional mapping and or association genetics and (d) implement a molecular breeding strategy such as MAS or more complex genomic approaches such as marker assisted recurrent selection and genomic selection (Mir et al. 2012) to combine genes and QTLs.

Other *omics* including functional genomics, genetic engineering, transcriptome profiling, proteomics and metabolomics have also shed light on heat tolerance in crops. Metabolic changes underpin plant development and responses to applied stresses, and this metabolic information reflects biological endpoints more accurately than transcript or protein analysis (Reddy et al. 2012). Metabolic profiling of *Arabidopsis* plants subjected to abiotic stresses has provided indicators that may enhance the development of heat tolerant cultivars in future.

12.8 Conclusions and Prospects

High temperature causing yield loss is expected to increase in frequency across the world and high-temperature stress, particularly during reproductive development, can reduce crop yield. High-temperature stress also has a strong influence on grain quality particularly milling characteristics in cereals. However, the timing, duration and intensity of high-temperature stress will determine its impact on crop yield and quality. Both specific and more general approaches are available to target various traits of crop growth and development. The adverse effect of high-temperature stress can be minimized by growing heat-tolerant genotypes with suitable agronomic practices. Screening for tolerance to temperature stresses has identified many promising sources of tolerance to high temperature in cereals, cotton, pulses and oil seed crops. However, research into assimilate partitioning and the establishment of high-throughput field based phenotyping will be critical, particularly if breeders are to take advantage of recent advances in genotyping technology.

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Chapter 13
QTLs for Genetic Improvement Under Global Climate Changes

Ramón Molina-Bravo and Alejandro Zamora-Meléndez

In loving memory of Raúl Molina Galaz
a father, a mentor, a loving and witty friend.
Your remembrance will forever be imprinted
in this work, and in the hearts of your family.

–Ramón Molina-Bravo

Abstract As the threat of climate change rises, breeders and scientists are facing new challenges in cultivar development. Many traits are becoming even more important in the changing environment, in particular, heat stress, changes in flowering behavior and unexpected pathogen infections. New cultivars will have to be equipped with adequate biotic resistances, abiotic tolerances and flowering requirements, which are largely quantitatively inherited, complex traits. Quantitative trait loci analysis is still the method of choice for identifying significant regions associated with complex traits. With up-and-coming, complementary technology, QTL discovery and introgressions of multi-gene traits are becoming a possibility in breeding cultivars in the changing environment. Herein, we discuss recent advancements in protocols for assessing these complex traits, and the associated QTLs to incorporate into innovative breeding strategies. Certain emphasis is made on woody perennial crops, as they present a greater challenge due to their longer life cycles compared to annual crops. New technologies are surging in, and are demonstrating new variations and augmentations to QTL discovery and introgression of multi-genes, such as fast-track breeding, and genomic selection.

Keywords Chilling requirement • Disease resistance • Flowering time • Heat tolerance • Quantitative traits • Woody perennials

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13.1 Introduction

Climatic models predict that the earth is expected to change in such a way that there will be increased temperatures and increased solar radiation (Lv et al. 2013; Stocker et al. 2013). Daily maximum temperature is expected to increase steadily, such that there will be an overall increase of 4 °C by the end of the century (Hall 2013; Vose et al. 2005). This presents a startling scenario for all plant production, from forage and seed, to fruit and nut, to fiber and ornamentals. Several crops are expected to experience earlier flowering, yield loss and growth reduction due to climate change, and because cultivar release occurs in terms of decades, breeders must focus on developing material that can endure new climatic conditions. Indeed, global climate change has resulted in severe yield losses worldwide and is threatening food security for the future (Lv et al. 2013). Coupled with an increasing population, new cultivars are expected to be highly productive under hotter temperatures, less water, insufficient chilling hours and exposure to new pathogens to meet demands. Breeders will depend on molecular tools to assist in the selection and evaluation process in annual crops, and even more so in perennial crops with long life cycles, such as fruit and nut crops. These latter crops, although usually not a staple food source, are an important source of income for many farm families, and because they tend to be perennial, yield loss or even whole-plant death poses an important economic threat. Quantitative trait loci analysis is the method of choice for identifying chromosome segments and candidate genes associated with complex traits (Argyris et al. 2010). New advances and improvements in current molecular tools and meta-analyses are showing high potential in keeping up with the current trends in climate change.

This chapter focuses on QTLs for three traits under the climate change threat; heat tolerance, flowering time and new pathogen resistance. Current advancements in crop improvement and technology are discussed. These new advancements can become tools to accelerate breeding, and develop cultivars that can tolerate these new conditions. Many cellular and physiological responses occur under changing climatic conditions, mostly in response to heat stress, and breeders will have to consider the introgression of heat stress-related traits. Breeders must also consider plant responses to flowering under warmer conditions. This is especially important for temperate climate, woody perennials that require chilling and heat exposure in order to break dormancy in the spring (Citadin et al. 2001; Westwood 1993). Lastly, new environmental conditions will inevitably follow new pathogenic threats. The effects of changes in temperature and precipitation have already been shown to have an effect on pathogen distribution (Garrett et al. 2013; Kriss et al. 2012). To conclude, new technologies that augment QTL detection and analysis are explored.

13.2 Heat Tolerance and Heat Stress Response

Heat stress presents a myriad of cellular responses, and is usually confounded by other abiotic stresses such as drought and salt stress. Tolerance to heat in plants is described by two commonly used terms: thermotolerance, and heat tolerance (HT).

Thermotolerance implies the involvement of heat shock proteins and their related genes and gene products in response to elevated temperatures (Nieto-Sotelo et al. 2002; Qu et al. 2013). Heat tolerance describes the ability to withstand physiological damage under elevated temperatures; for example, non-tolerant cultivars will have less viable pollen than tolerant cultivars.

Heat stress can lead to physiological injuries such as scorching of leaves and stems, leaf cupping, leaf abscission, inhibition of growth, shortened life span and fruit damage (Adams et al. 2001; Kadir and Sidhu 2006; Vollenweider and Günthardt-Goerg 2005). See Fig. 13.1g–i. Exposure to heat reduces the plant's assimilation rates and thus reduces growth and total dry weight (Wahid et al. 2007). Many changes occur at the inter- and intracellular level in response to elevated temperatures, even at 5 °C above optimum ambient temperature (Fernandez and Pritts 1994; Guy 1999). This array of changes in response to supraoptimal temperatures includes reorganization of cellular structures, transcription of heat shock proteins (HSPs) and even the synthesis of several protective compounds such as phytohormones and antioxidants (Venkateswarlu and

Fig. 13.1 Phenotypic alterations in tomato (*Solanum lycopersicum*) flowers **a–d** and raspberry (*Rubus idaeus*; **e–i**) exposed to heat stress (hs). The hs conditions (36 °C/26 °C, day/night) expose abnormal anthers and style elongation (**c**, **d**), compared to control treatment conditions (ct; 26 °C/19 °C, day/night; **a**, **b**) in flower buds and opened flowers of the tomato cultivar, Saladette. Raspberry cultivars and selections in hs conditions at the Sandhills Research Station (SHRS), Jackson Springs, NC, USA, and in non-hs conditions at the Mountain Horticultural Crops Research & Extension Center (MREC), Mills River, NC, USA [**e–i**; maximum temperatures range is 30–35 °C between July and August at SHRS, and 21–32 °C at the MREC (State Climate Office of North Carolina, 2009, 2014)]. Moderate **f** to severe **e** leaf cupping of raspberry selections due to hs conditions at the SHRS; heat tolerant Mandarin exhibits vigorous growth (**g**, *solid arrow*) and fruit production **h** versus non-heat tolerant selections (**g**, *dashed arrow*); fruit produced by North Carolina selection, NC548, yields smaller fruit when grown in hs conditions at the SHRS (**i**, *right*), than in non-hs conditions at the MREC (**i**, *left*) (Source: Figures **a–d** taken from Giorno et al. (2013); Figures **e–i** courtesy of the North Carolina State University *Rubus* breeding program)

Shanker 2012; Wahid et al. 2007). Enzyme productivity is also affected by heat stress. Most notably, heat stress can cause irreversible damage to enzymes in the thylakoid membranes and chloroplast stroma involved in the Calvin-Benson cycle, including Rubisco (Ribulose 1,5-bisphosphate carboxylase) and Rubisco activase, hence compromising normal photosynthesis (Crafts-Brandner and Law 2000; Maestri et al. 2002).

The inhibition of net photosynthesis in response to temperature can be attributed to a decrease in the amount of active Rubisco, both in C3 and C4 plants (Salvucci and Crafts-Brandner 2004). Membrane fluidity is also affected during warmer conditions, but the membrane itself is also involved in complex mechanisms that sense elevated ambient temperatures. Sets of complicated sensors are positioned in various cellular compartments; when membrane fluidity increases, there is an activation of lipid-based signaling cascades, an increased Ca2+ influx, and cytoskeletal reorganization (Bita and Gerats 2013). Increased temperatures lead to a state of dehydration, which disrupts cellular membranes in such a way that they become permeable to ions due to increased solubilization and peroxidation of membrane lipids (Iba 2002; Yang et al. 1996) and photosynthetic membranes exposed to heat result in the stacking and swelling of grana (Gounaris et al. 1984). Other responses to heat stress include whole organs as well. In terms of reproductive tissue damage, lowered photosynthetic rates result in a limitation of available resources. Heat stress in terms of reproduction and flowering inevitably affect plant productivity. The early stages of flower bud formation is most vulnerable to heat stress (Bita and Gerats 2013; Hedhly 2011; Nava et al. 2009). The damage observed is variable, and can include male gametophyte abnormalities, delayed growth of the female gametophyte, and abortion of the tapetal cells, which leads to pollen sterility (Nava et al. 2009; Oshino et al. 2007; Parish et al. 2013; Sakata and Higashitani 2008). If fertilization occurs, the continued exposure to heat stress can impede further embryo as well as seed development (Barnabás et al. 2008). Grain filling is frequently used as a means to assess HT in field evaluations and in QTL studies (Farooq et al. 2011; Tiwari et al. 2013; Yang et al. 2002; Ye et al. 2011). There is evidence that the speed of the current climate change is overwhelming some plant species' ability to adapt. In maize, climate warming has reduced overall yield by 3.8 % compared to the theoretical maximum, mostly due to elevated temperatures, and not precipitation (Lobell et al. 2011; Shaw and Osborne 2011). Similarly in *Arabidopsis thaliana*, Wilczek et al. (2014) tested the hypothesis of lagging adaptation to climate change using common garden experiments. They found that immigrant plants from historically-warmer climates outperformed native plants in every site, but especially at the northern range limit of the species distribution in Finland. These results have direct implications for current breeding efforts and for the conservation of wild species and crop relatives. Breeders of temperate or high elevation crops should incorporate adaptation from accessions from historically warmer climates and this is particularly relevant to woody perennial crops.

13.2.1 Screening Methods and QTLs

For QTL analyses of plant HT, recognizing and understanding these responses is essential for phenotyping the trait in study populations. Breeding for stress tolerance will require effective and efficient screening procedures, as well as the identification of key genes in donor and study material. Sequencing analysis has undergone a revolution in data acquisition. Next-generation sequencing can generate a massive amount of data, where the challenge is in bioinformatics to filter and select relevant sequences; cost and time is not necessarily an issue (Egan et al. 2012). However, there are no standardized methods for phenotyping HT, or for many other complex traits for that matter. Thus, developing and applying screening methods for QTL analyses can be challenging. Additionally, analyzing complex traits for quantitative analysis requires the evaluation of large populations in multi-year trials. This implies that screening methods should process and measure the trait of interest in numerous individuals quickly and effectively.

Depending on the screening method, the discovery of QTLs can be difficult or uncertain when dealing with complex traits. Since the trait itself results in multiple plant responses, QTL regions may be associated only with a particular response or may not be directly associated with HT, hence the discovery of minor QTLs. Nevertheless, many methods have been used to locate genomic regions associated with HT in plants. This section discusses screening methods in detail, since accurate phenotyping is important for finding meaningful QTL regions (Collard et al. 2005).

Because of the complex interactions and responses to heat stress, a single phenotyping method for HT can reveal loci specifically associated with the method of detection. To reveal QTLs involved in heat stress response, a combination of phenotyping methods has been used in numerous types of crops including cereals, legumes, fruit and forestry crops (Ali et al. 2013; Ismail and Hall 1999; Molina-Bravo et al. 2011; Percival and Sheriffs 2002; Srinivasan et al. 1996; Stafne et al. 2001; Stoddard et al. 2006; Wahid and Shabbir 2005; Weng and Lai 2005; Willits and Peet 2001; Yamada et al. 1996; Yang et al. 1996). Screening methods usually measure HT by evaluating yield or yield components, visual assessment, chlorophyll fluorescence or membrane permeability. Due to the high number of individuals in study populations, a screening method should be able to measure the trait efficiently, and accurately in a high throughput manner. This is especially the case in QTL analysis, where experiments require numerous individuals or numerous populations. The analysis can then be further enriched with association mapping, meta-QTL analysis or fine mapping.

13.2.1.1 Stress Indices

In agronomic crops, stress indices have been used to evaluate high temperature tolerance in plants, such as the thermal stress index in cotton, which is based on foliar temperatures (Burke et al. 1990). Canopy temperature depression (CTP) is

positively correlated to grain yield in wheat, and is recommended as an indicator of tolerant genotypes (Pradhan et al. 2012). Many studies have used a yield-based approach as an indicator or index of HT (Burke et al. 1990; Farooq et al. 2011; Fokar et al. 1998; Lin et al. 2010; Porch 2006). In many cereal crops, grain weight and grain number are affected by heat, namely there is a negative correlation between grain number and temperature, i.e. as the temperature increases, grain number decreases during flowering and grain filling (Fokar et al. 1998; Spiertz et al. 2006; Wardlaw 1994; Zhao et al. 2007). The geometric mean (GM) and the stress tolerance index (STI) have been used to compare performance across environments (Fernandez 1992; Ramirez-Vallejo and Kelly 1998). The stress susceptibility index (SSI) is a ratio of the genotypic performance under stress and non-stress conditions (Fischer and Maurer 1978) and correlates well with yield and canopy temperatures in wheat (Rashid et al. 1999). Other indices define stress tolerance as the difference in yield between stress and no stress-environments such as the mean productivity index (MP) (Rosielle and Hamblin 1981). Studies combine these indices and compare their accuracy for selection as a means to better screen for tolerant genotypes (Khodarahmpour et al. 2010; Porch 2006; Ramirez-Vallejo and Kelly 1998). In woody perennials however, yield itself is confounded by environmental factors other than heat stress, such as pollination by insects to stimulate fruit production, fulfilling chilling requirements during the winter, virus infections and other factors (Westwood 1993). Yield evaluations in study populations are also a challenge in woody perennials because measurements are usually based on very few individual plants per genotype. Therefore, yield-based indices should be used with caution when considering crops such as stone fruits, nuts or berries. Alternatively, HT is screened using visual assessment or vigor indices. Most modern red raspberry cultivars are adapted to cool climates (Hall et al. 2009; Jennings 1988), but breeding programs have successfully bred *Rubus* cultivars tolerant to the warm regions of the south and southeastern Unites States by incorporating species from warm temperate and/or subtropical regions of Asia through visual assessment (Ballington and Fernandez 2008; Hall et al. 2009; Williams 1950). Some of these cultivars include Van Fleet (*R. kuntzeanus* × Cuthbert 1924), Dormanred (*R. parvifolius* × Dorsett 1979) and Mandarin [(*R. parvifolius* × Taylor) × Newburgh] (Ballington and Fernandez 2008; Hall et al. 2009). See Fig. 13.1g.

13.2.1.2 Physiological and Cellular Responses

Tissue senescence is a typical stress symptom in plants, which is a manifestation of membrane damage due to increased fluidity of lipids, lipid peroxidation and protein degradation (Savchenko et al. 2002). Therefore, the level of saturation of fats is indirectly associated with heat tolerance as this affects the fluidity of membranes. Increasing the level of saturation yields better membrane stability and therefore enhances HT (Larkindale et al. 2005). Lipid composition can be used as an indicator of HT. Fatty acid composition in plants can be studied by separation through high pressure liquid chromatography (HPLC) and mass spectrometry (Djafi et al. 2013),

and although this may not be a practical screening method, fatty acid composition is important in HT (Iba 2002; Upchurch 2008). Lipid peroxidation of cell membranes is a response to heat stress. In creeping bentgrass (*Agrostis palustris*), lipid peroxidation product content, i.e. malondialdehyde, is measured through enzymatic assays and distinguishes between tolerant and non-tolerant genotypes (Liu and Huang 2000).

Accumulation of osmoprotectants is also an adaptive mechanism of HT in plants. Proline, glycinebetaine (GB) and soluble sugars accumulate in the plant cell to regulate osmotic activities, stabilize the cell membrane and to buffer the cellular redox potential (Farooq et al. 2008). Yang et al. (1996) developed maize near isogenic lines (NILs) that are homozygous for the GB-containing and GB-deficient gene (Bet1/Bet1 and bet1/bet1, respectively). GB-containing NILs exhibit less membrane injury than GB-deficient NILs, and Bet1/Bet1 plants showed higher concentrations of GB. Secondary metabolites are also implicated in thermal stress. For example, anthocyanin accumulation decreases leaf osmotic potential under heat stress, which results in increased water uptake and reduced water loss (Chalker-Scott 2002; Wahid et al. 2007). Carotenoids function as cell membrane protectants; xanthophylls and other terpenoids, such as isoprene or tocopherol, stabilize and protect thylakoid membrane lipids (Camejo et al. 2006; Velikova et al. 2005). Similarly in *Arabidopsis* experiments, plants overexpressing the gene that encodes β-carotene hydroxylase show a greater tolerance to increased temperatures, most likely by preventing oxidative damage to membranes (Maestri et al. 2002). Thus, measuring secondary metabolite content is an additional method of measuring HT.

Other ways of measuring HT that are not yield-based include pollen viability and anther dehiscence, since pollen damage is a response to heat stress (Bita and Gerats 2013; Oshino et al. 2007; Parish et al. 2013; Sakata and Higashitani 2008). Anther abnormalities and style elongation are also physiological responses to heat stress (Giorno et al. 2013). See Fig. 13.1a–d. In rice, plants that are heat tolerant dehisce more easily than susceptible individuals under high-temperature conditions (Jagadish et al. 2010b; Prasad et al. 2006). However, a disadvantage to this method is that the technique can only be applied during flowering, thus the time-frame for screening is narrow, and requires plants to reach maturity; a considerable time for plants with long juvenile periods.

At the cellular level, measuring membrane integrity, enzyme viability, chlorophyll fluorescence (to measure electron transport rate) and gas exchange (to measure photosynthesis) have all been used successfully to screen for HT in field-grown plants (Cottee et al. 2010; Crafts-Brandner and Law 2000; Molina-Bravo et al. 2011; Porcar-Castell et al. 2014; Ranney and Peet 1994; Srinivasan et al. 1996; ur Rahman et al. 2004; Yamada et al. 1996; Yang et al. 1996). Measuring chlorophyll content has also been proposed as a high-throughput screening method for tolerance to heat, since damage to thylakoid membranes is closely associated with loss of chlorophyll (Shah et al. 2011). In methods that measure membrane leakage and chlorophyll fluorescence, warm environmental conditions, and sometimes a heat-shock treatment is necessary to be able to distinguish tolerant genotypes (Blum and Ebercon 1981; Molina-Bravo et al. 2011; ur Rahman et al. 2004; Weng and Lai

2005; Yamada et al. 1996). This would imply that additional steps should be considered to reveal cell injury and should be regarded with caution. A thorough characterization of HT involves measuring a combination of parameters. In a Nagina22 rice mutant, pollen viability, spikelet fertility, chlorophyll and carotenoid content, and percent yield are higher than in non-HT genotypes (Poli et al. 2013). But rapid screening devices are more advantageous and feasible for handling numerous individuals. Some of these devices include those that measure chlorophyll fluorescence, enzyme integrity and membrane leakage. When screening large and genetically-diverse populations, however, measuring photosynthesis, fatty acid composition, gene expression, yield or yield components is not practical; instead, these methods can be used strategically for validation (Jagadish et al. 2010b).

13.2.2 QTLs for Heat Tolerance

The numerous methods mentioned above are applied to QTL analysis to reveal significant regions associated with HT. Several studies combine these methods to better screen for heat tolerant genotypes and for QTL detection (Poli et al. 2013; Trijatmiko et al. 2014). Cereal crops are perhaps the best studied in terms of QTL regions associated with HT. Multiple stable QTL for HT are found in wheat and maize (Ali et al. 2013; Lei et al. 2013; Paliwal et al. 2012; Pinto et al. 2010; Poli et al. 2013; Tiwari et al. 2012; Trijatmiko et al. 2014; Vijayalakshmi et al. 2010; Wei et al. 2013; Yang et al. 2002; Ye et al. 2011). In rice, numerous QTLs associated with HT have been identified at both booting and flowering stages in various rice populations, and QTLs were found on all chromosomes except 6 and 7 (Buu et al. 2014; Cao et al. 2003; Jagadish et al. 2010a; Xiao et al. 2011; Ye et al. 2010; Zhang et al. 2009). Fine mapping of these QTLs has also been proposed (Ye et al. 2011). In wheat, QTLs are found on chromosomes 2B, 7B and 7D in a mapping population phenotyped by HSI, thousand-grain weight, HSI for grain-fill duration and CTP, and explain as much as 20.34 % of the variation (Paliwal et al. 2012). Although other approaches have been used, the majority of these studies use yield-based methods for phenotyping HT. An extensive review of QTLs in cereals under climate change threat is available and discusses these subjects in great detail (Dwiyanti and Yamada 2013).

13.3 Flowering Time

Flowering time is an economically-important trait, especially in the context of global climate change. There is a plethora of information available on the flowering regulation in annual crops, particularly in *Arabidopsis*. Numerous QTLs have been identified, and a thorough review has been written, where specific genes and putative functions are discussed (Dwiyanti and Yamada 2013). Using molecular

tools for breeding specific flowering time is perhaps more important in perennial crops due to the long juvenile periods (breeding is more time consuming than in annual crops).

Herein, the focus will be on the flowering regulation in woody perennials, since this subject has been discussed only marginally regarding QTLs and global climate change. For flowering to occur in woody perennials in temperate and cold climates, buds undergo a dormancy period, where they differentiate from vegetative primordial cells to reproductive buds (Westwood 1993). This dormancy period is broken when buds are exposed to low temperatures (usually 0–7 °C) for a period of many (accumulated) hours; this is known as the chilling requirement (CR) Carter et al. (2006). Additionally, an accumulation of growing degree hours or days, called the heat requirement (HR), is also necessary to break dormancy.

The term dormancy has undergone several definitions, but the working definition currently used is the temporary suspension of visible growth of any plant structure containing a meristem (Lang 1987). The terms *temporary* and *meristem* are included to signify that growth can resume and that growth arises from undifferentiated cell division. From this term, Greek prefixes are added to describe specific types of dormancy such as endodormacy, paradormancy and ecodormancy. Paradormancy refers to the suspension of growth due to internal morphological blocks other than the affected tissue, such as apical dominance, while ecodormancy refers to the suspension due to inadequate environmental factors, such as a lack of nutrients (Lang 1987). Hereafter, we will only discuss endodormancy, which is dormancy due to internal physiological blocks (Lang 1987; Westwood 1993). These physiological blocks prevent growth under unfavorable environmental conditions and terminate after CRs and HRs have been met (Westwood 1993). For many woody perennial crops, CRs and HRs can vary, depending on the species or cultivar. There is variability in both of these requirements even among closely-related genotypes (Fan et al. 2010; Molina-Bravo et al. 2014; Warmund and Krumme 2005; Westwood 1993).

Many important horticultural deciduous species require an accumulation of chilling hours in order to break bud dormancy, including *Vitis* (grape), *Ribes* (currant), *Carya* (pecan), *Malus* (apple), *Pyrus* (pear), *Prunus* (peach, almond, plum, apricot, cherry) and *Rubus* (raspberry, blackberry). However, chilling models show that accumulated temperatures are decreasing over time throughout the world (Luedeling and Brown 2011; Luedeling et al. 2009, 2011). Under this environmental change, breeders need to consider yield losses due to insufficient chilling as well as replacing current cultivars with low-chilling cultivars (Topp et al. 2008). Middle-latitude temperate species of deciduous fruits and nuts are adapted to winters that fluctuate between cold and warm temperatures by having high chilling requirements to break bud dormancy (Westwood 1993). In the event of frost, winter injury can be avoided because reproductive growth will not occur in midwinter under favorable growth conditions. If warmer winters are to be expected, low chilling cultivars should be bred for because plants are able to break dormancy and grow after a short exposure to cold, as typically occur in warmer regions. High-chilling cultivars are suitable when winter conditions are irregular.

13.3.1 Chilling and Heat Requirement Models for Flowering Time

The chilling requirement is measured using mathematical models that require monitoring temperatures during dormancy. Many models have arisen through modifications of three main models: the chilling-hour model, the Utah chilling model and the dynamic model (Bennett 1949; Fishman et al. 1987; Richardson et al. 1974). The chilling-hour model assumes all air temperatures 0–7 °C are equally effective (Bennett 1949). However, further insight into tree-chilling response revealed that warm temperatures have a negative effect on chilling accumulation (Campbell 1955). This prompted the development of a weighted, unit-based model—the Utah chilling model—where temperatures below 0 °C and above 7.2 °C are assigned partial chilling units (Richardson et al. 1974). Temperatures above or below a certain limit can also reverse the effect of chilling in this model (Cesaraccio et al. 2004). In contrast, the dynamic model has a two-step process, and assumes a biochemical basis for endodormancy release (Fishman et al. 1987). The dynamic model states that the first step is the formation of a precursor for breaking dormancy, high temperatures reverse the effect, while low temperature promote the effect. The second step is an irreversible transition from an unstable precursor to a stable dormancy-breaking factor and chilling requirements are calculated as chilling portions. Some modified versions of the Utah chilling model include the North Carolina model used for apples, which proposes a broader range of effective temperatures and incorporates a greater negative effect when temperatures exceed 21 °C (Shaltout and Unrath 1983). More recently, a modified chilling-hour model is used for blackberry, where time of chilling inception occurs at the first incidence of −2.2 °C (Yazzetti and Clark 2001), and for highbush blueberry, where the model accumulates units at temperatures below 12.5 °C, and 7 °C (Norvell and Moore 1982; Spiers et al. 2006). For raspberry, the current model assumes temperatures below 5.6 °C = 1 chill unit, and those above 13 °C = −1 chill unit (Dale et al. 2003). Two modified Utah chilling models are used for blackberry with weighted chilling units that encompass adjusted temperature ranges and different times of chilling inception (Warmund and Krumme 2005).

Not all models are equally as effective. For example, the Utah chilling model is not accurate in *Rubus* (Warmund and Krumme 2005). This is attributed to the lack of an appropriate parameter to determine the point of inception, therefore some models need adjustments depending on the genus. Other comparisons among models have shown a strong correlation in estimates between the dynamic and Utah chilling models, although Utah model calculates different CRs depending on the intensity of the winter (Erez et al. 1990; Ruiz et al. 2007). The dynamic model best determines CRs in warm winter regions, such as Israel, but behaves similarly in temperate regions (Alburquerque et al. 2008; Erez et al. 1988; Zhang and Taylor 2011). Therefore, the dynamic model is the most robust across climates, and is often used in global climate change studies (Luedeling and Brown 2011). Campoy et al. (2011a) and Luedeling (2012) have written comprehensive reviews on the comparison and performance of chilling models, and their effect on climate change.

Flowering time complexity is further augmented because CR is not the only determinant factor. HRs also determine budbreak in woody perennials, although whether this requirement comes post-completion to chilling is still unclear (Citadin et al. 2001; Fan et al. 2010; Richardson et al. 1974). To further complicate the matter, extended chilling can reduce the HR (Citadin et al. 2001; Harrington et al. 2010; Scalabrelli and Couvillon 1986). In several Rosaceae species, extended chilling, in many cases, accounts for 90 % of the HR variation, that is to say, that budbreak and bloom dates are mostly determined by CR (Couvillon and Erez 1985). In *Prunus* species, CR is a major determinant factor of bloom date (Alburquerque et al. 2008; Ruiz et al. 2007; Sánchez-Pérez et al. 2012), while in *Malus*, each effect has a different impact depending on the genetic background (Celton et al. 2011). In pecan, budbreak is under the interactive control of heating and of chilling; the required heat for budbreak is inversely proportional to chill accumulation but budbreak may occur without chilling if there is sufficient accumulated heating degree days (Sparks 1993). Ruiz et al. (2007) reported a negative correlation between the two factors in apricot, while Scorza and Okie (1990) found peach genotypes with low CRs, but have late blooming dates. HRs have been measured based on the growing degree hour (GDH) model proposed by Richardson et al. (1975). Although the model has been widely used, it only accounts for the heat accumulation after endodormancy release (Alburquerque et al. 2008; Citadin et al. 2001; Ruiz et al. 2007). The accumulation of GDH is determined by a modified cosine curve defined by three cardinal temperatures in response to growth: a base temperature of 4 °C, an optimum of 25 °C and a critical temperature of 36 °C (Anderson et al. 1986; Richardson et al. 1974). Current experiments on HR models for raspberry determined that the best fit are a linear model and an asymmetric curvilinear relationship (ASYMCUR) model (Anderson et al. 1986; Black et al. 2008). For the linear model, the base and optimum temperatures are 6 °C and 25 °C, and for the ASYMCUR model, the base and optimum temperatures are 4 °C and 27 °C, respectively.

13.3.2 QTLs for Flowering Time in Woody Perennial Crops

One of the best-studied systems for flowering time in fruit crops is the peach and other members in the genus. A recent published review summarizes the advancements in almond and other *Prunus* species (Sánchez-Pérez et al. 2014). Genetic and genomic studies are inching their way towards a better understanding of the mechanisms that control flowering time in woody species. The trait is described as polygenic (Anderson and Seeley 1993; Sánchez-Pérez et al. 2007), but in Tardy Nonpareil progenies, a late-blooming phenotype segregates in a bimodal fashion, suggesting the involvement of a major gene (Kester 1965). This gene is confirmed as Lb and is a major gene that maps onto *Prunus* LG4 (PLG4); Ballester et al. (2001). Another major contributor to understanding genetic factors of flowering time in *Prunus* is the discovery of a feral peach grown in central Mexico, known as the *evergrowing* or *evg* mutant (previously known as Evergreen), which, as its name suggests, has terminal

buds that are continuously growing, and never enter dormancy but still set flowering buds (Rodriguez-A et al. 1994). The progeny from crosses between the evg mutant and deciduous peaches show the involvement of a single recessive gene, called EVERGROWING or EVG, that gives rise to the evergrowing phenotype (Rodriguez-A et al. 1994). EVG maps onto PLG1, and sequencing analyses show that EVG is a cluster of six Dormancy Associated MADS-box (DAM) genes (Bielenberg et al. 2008; Wang et al. 2002). In the evg mutant, there is a large deletion of four out of the six DAM genes, and although two of these DAM genes are present, none of the six DAM genes are expressed (Bielenberg et al. 2008).

Numerous QTL studies identified significant regions associated with CR or flowering time on PLG1 and PLG4 in various *Prunus* species, including peach (Fan et al. 2010; Zhebentyayeva et al. 2014), apricot (Olukolu et al. 2009), almond (Ballester et al. 2001; Sánchez-Pérez et al. 2012), and sweet cherry (Castède et al. 2014). Not surprisingly, several of these QTLs co-localize or are near EVG or Lb (Silva et al. 2005). Many QTLs are present throughout different linkage groups in several *Prunus* species associated with flowering time; all eight groups except PLG8 in four populations of peach (Dirlewanger et al. 1999; Quilot et al. 2004; Fan et al. 2010), PLG1, 5 and 7 in apricot (Campoy et al. 2011b; Salazar et al. 2013), and PLG1, 2, and 4 in sweet and sour cherry (Castède et al. 2014; Dirlewanger et al. 2012; Wang et al. 2000). Perhaps these studies reflect the polygenic nature of the trait. Nevertheless, QTLs co-localized onto EVG and Lb on PLG1 and PLG4, respectively, are major contributors of the CR and flowering time traits. In some cases, these QTLs explain a high percentage of the variability in *Prunus*, but more so in almond in the case of the Lb gene (Castède et al. 2014; Dirlewanger et al. 2012; Fan et al. 2010; Olukolu et al. 2009; Sánchez-Pérez et al. 2007, 2012; Zhebentyayeva et al. 2014). QTL studies on flowering time traits have been identified in other Rosaceae species.

In apple, QTLs for time of budbreak and CR are on various *Malus* linkage groups (MLG), where a major QTL is on MLG9 (Celton et al. 2011; Conner et al. 1998; Segura et al. 2007; van Dyk et al. 2010). A second stable QTL is also on MLG8 (Celton et al. 2011; Segura et al. 2007). In raspberry, few studies have looked at CR, and floral development to ripening traits. QTLs are on *Rubus* linkage groups (RubLGs) 1, 4, 5, and 6 for CR; and on RubLG1 [chromosome6 (C6) in Graham et al. (2009)], RubLG3 (C2), RubLG5 (C5), and RubLG6 (C3) for floral development to ripening (Graham et al. 2009; Molina-Bravo et al. 2014). Studies on QTLs linked to CR and flowering in other small fruits include blackcurrant (*Ribes nigrum*), grape (*Vitis vinifera*), and an interspecific diploid blueberry [(*Vaccinium darrowii* × *V. corymbosum*) × *V. corymbosum*]. In blackcurrant, the most significant QTL associated with budbreak is on *Ribes* linkage group 3 (RibLG3), while other significant QTLs for leaf and flower budbreak map onto RibLG1, 7 and 8 (Brennan et al. 2008). In grape, there are six QTLs; two associated with budbreak on *Vitis* chromosomes (VC) 4 and 19, two on VC7 and 14 for flowering, and two on VC16 and 18 for veraison, i.e. onset of ripening (Duchêne et al. 2012). One major QTL for growth cessation maps onto *Vitis* LG9 under greenhouse conditions, and an additional major QTL on *Vitis* LG13 under field conditions (Garris et al. 2009). In diploid

blueberry, QTLs for CR are found on *Vaccinium* linkage group (VLG) 6 and VLG8. For a more detailed summary of these QTLs, see Table 13.1.

13.3.3 MADS-Box Like Genes

The best understood woody plant genetic model for bud dormancy has been described in poplar (*Populus*); Cooke et al. (2012) have written an extensive review, which includes other woody perennials. For *Prunus* species, however, many gene studies have been reported and contribute to the understanding of endodormancy and budbreak in economically-important fruit crops. Since the identification of the EVG gene cluster (Bielenberg et al. 2004, 2008), researchers have focused on the functionality and expression of the DAM genes (a total of six genes, DAM1 through DAM6) within the cluster as a way to understand the genetic control of endodormacy. DAM genes are phylogenetically similar to the StMADS11 clade MADS box genes in *Arabidopsis*, which include the SHORT VEGETATIVE PHASE (SVP) and AGAMOUS-LIKE24 (AGL24) transcription factors (Yamane et al. 2008). In *Arabidopsis*, SVP inhibits flowering by downregulating FLOWERING LOCUS T (FT), while AGL24 promotes flowering by upregulating LEAFY (*LFY*) (Hartmann et al. 2000; Michaels et al. 2003). Similar to SVP, peach DAM1, DAM2, and DAM4 transcription factors are correlated with terminal bud formation (bud set) and growth cessation (Li et al. 2009), while DAM3, DAM5, and DAM6 are correlated with dormancy and DAM5 and DAM6 with quantitative repression of budbreak (Jiménez et al. 2010; Li et al. 2009). DAM genes are also expressed in both vegetative and floral peach buds, and DAM5 and DAM6 are upregulated by short days (i.e. repression of budbreak during short days) but downregulated by low temperature (Jiménez et al. 2010; Leida et al. 2012; Li et al. 2009; Yamane et al. 2011). A similar situation has been observed in Japanese apricot (*Prunus mume*), where apricot DAM genes are preferentially expressed in dormant buds, and all six DAM genes are downregulated during dormancy release of lateral vegetative buds (Yamane 2014). All six *P. mume* DAM genes are downregulated during winter and during prolonged cold treatments (Sasaki et al. 2011). All of the DAM genes in Japanese apricot share amino acid similarity, including an amphiphilic repression motif, known to act as a repression domain, at the carboxyl-terminal end, suggesting that all *P. mume* DAM genes may act as transcriptional repressors (Ohta et al. 2001; Sasaki et al. 2011). Other MADS-box like genes with similar expression patters have been described in temperate fruit crops. In raspberry, a RiMADS1 gene, which bears sequence similarity to the *Prunus* DAM6 gene, encodes an SVP MADS-box transcription factor that is downregulated during dormancy release (Graham et al. 2009; Mazzitelli et al. 2007). Interestingly, RiMADS1 maps onto the upper portion of RubLG5, and QTLs for floral development and CR are associated with the upper portion of RubLG5, corroborating the involvement of this region during flowering and endodormancy release (Graham et al. 2009; Molina-Bravo et al. 2014).

Table 13.1 Summary of QTLs associated with flowering traits in major woody perennial crops

Species	Trait	Population	No. of QTLs	Linkage group or chromosome	% explained variation	Source
Almond	Bloom date	(Tardy Nonpareil × Tuono) × Desmayo Langueta	8	1, 4, 6, 7	8.4–58.3	Sánchez-Pérez et al. (2012)
Prunus dulcis	Chilling requirement	(Tardy Nonpareil × Tuono) × Desmayo Langueta	8	1, 3, 4, 7	20.2–99.9	Sánchez-Pérez et al. (2012)
	Heat requirement					
		(Tardy Nonpareil × Tuono) × Desmayo Langueta	3	2, 7	58.9–82.0	Sánchez-Pérez et al. (2012)
Almond × peach	Bloom date	Texas × earlygold	2	2, 7	20.5–27.4	Dirlewanger et al. (1999)
P. dulcis × *persica*	Bloom date	Texas × earlygold	5	4	32.1–35.1	Verde et al. (2002)
	Bloom date	Texas × earlygold	4	1, 4, 6, 7		Silva et al. (2005)
Apple	Vegetative budbreak	Golden Delicious × Anna, and Sharpe's Early × Anna	1	9	4.8–44.6	Van Dyk et al. (2010)
Malus × *domestica*	Green point	Starkrimson × Granny Smith	8	6, 8, 9, 10, 12	15.2–27.1	Celton et al. (2011)
	Vegetative budbreak	Starkrimson × Granny Smith	7	2, 3, 5, 6, 10	9.9–26.4	Celton et al. (2011)
	Floral budbreak	Starkrimson × Granny Smith	7	6, 8, 12	8.7–23.6	Celton et al. (2011)
	Green point	X3263 × Belrène	10	1, 3, 9	5.1–37.5	Celton et al. (2011)
	Vegetative budbreak	X3263 × Belrène	9	1, 3, 9, 10, 15	5.5–14.9	Celton et al. (2011)
	Floral budbreak	X3263 × Belrène	8	1, 9, 12, 17	6.9–29.1	Celton et al. (2011)
	Bloom date	Starkrimson × Granny Smith	2	6, 8	34.0	Segura et al. (2007)

Apricot	Bloom date	Z701-1 × Palsteyn	7	1, 4, 7	9.0–51.6	Salazar et al. (2013)
P. armeniaca	Chilling requirement	A.1740 × perfection	12	1, 2, 5, 6, 7, 8	58.5–66.1	Olukolu et al. (2009)
	Flowering time	(Orange Red × Currot) × Currot	1	5	40.0–80.0	Campoy et al. (2011b)
Blackcurrant	Budbreak	SCRI S36/1/100 × EMRS B1834	2	3, 8	N/A	Brennan et al. (2008)
Ribes nigrum	Full leaf	SCRI S36/1/100 × EMRS B1834	3	1, 3, 7	N/A	Brennan et al. (2008)
	First flower	SCRI S36/1/100 × EMRS B1834	2	3, 8	N/A	Brennan et al. (2008)
	Full flower	SCRI S36/1/100 × EMRS B1834	1	1	N/A	Brennan et al. (2008)
Blueberry	Chilling requirement	Fla4B × (W85-20 × W85-23)	3	6, 8	14.6–18.6	Rowland et al. (2014)
Vaccinium darrowii × *V. corymbosum*	Critical photoperiod	*V. riparia* × Seyval	5	11, 13	88.0–96.6	Garris et al. (2009)
Grape						
Vitis riparia × *V.* hybrid	Budbreak	Riesling × Gewurztraminer	5	4, 19	8.3–18.7	Duchêne et al. (2012)
	Flowering	Riesling × Gewurztraminer	9	7, 14	13.1–28.0	Duchêne et al. (2012)
Vitis vinifera	Veraison	Riesling × Gewurztraminer	8	16, 18	13.2–20.6	Duchêne et al. (2012)
Peach	Bloom date	Contender × Fla.91-2C	23	1, 2, 4, 5, 6, 7	52.0–74.1	Fan et al. (2010)

(continued)

Table 13.1 (continued)

Species	Trait	Population	No. of QTLs	Linkage group or chromosome	% explained variation	Source
P. persica	Chilling requirement	Contender × Fla.91-2C	13	1, 4, 5, 6, 7, 8	50.2–56.3	Fan et al. (2010)
	Heat requirement	Contender × Fla.91-2C	3	1, 8	8.6–10.7	Fan et al. (2010)
	Bloom date	Bolero × OroA	1	7	9.2	Eduardo et al. (2010)
	Chilling requirement	Contender × Fla.91-2C	17	1, 2, 4, 5, 6, 7, 8	56.4–56.8	Zhebentyayeva et al. (2014)
	Bloom date	Contender × Fla.91-2C	29	All 8	52.4–76.4	Zhebentyayeva et al. (2014)
	Bloom date	Zéphyr × [(*P. davidiana* × Summergrand) × Sumergrand]	8	1, 2, 4, 5, 6	27.0–45.0	Quilot et al. (2004)
Raspberry	Chilling requirement	(*R. parvifolius* × Tulameen) × Qualicum	7	1, 4, 5, 6	15.3–65.8	Molina-Bravo et al. (2014)
Rubus idaeus	Budbreak to ripening	Glen Moy × Latham	27	1, 3, 5, 6	5.6–44.0	Graham et al. (2009)
Rose	Bloom date	H190 × *R. wichuraiana*	1	4	34.0	Hibrand-Saint Oyant et al. (2008)
Rosa wichuraiana						
Sweet cherry	Chilling requirement	Regina × Garnet	6	1, 2, 4, 6, 7	5.8–17.5	Castède et al. (2014)
P. avium	Heat requirement	Regina × Garnet	6	1, 3, 4, 6, 8	5.3–0.9	Castède et al. (2014)
	Flowering date	Regina × Garnet	11	1, 2, 3, 4, 5, 6, 7, 8	6.8–76.1	Castède et al. (2014)
	Bloom date	Regina × Lapins	5	1, 2, 4, 5, 8	3.3–47.0	Castède et al. (2014)

In Japanese pear (*Pyrus pyrofolia*), MADS13, a DAM-like gene, is upregulated towards dormancy establishment, and as in peach DAM5 and DAM6, is downregulated towards dormancy release (Saito et al. 2013). In kiwifruit (*Actinidia deliciosa*), SVP-like genes play distinct roles in dormancy and flowering: the *Actinidia* SVP1 gene is able to compensate for the loss of *SVP* in *Arabidopsis*, but is not highly similar to the peach DAM genes (Wu et al. 2012). DAM genes play such an important role in dormancy that a model for predicting CR in different cultivars based on gene expression has been proposed in peach: high-chill peach cultivars have higher expression of DAM5 and DAM6 than low-chill cultivars prior to chilling accumulation in floral buds (Leida et al. 2012). This model uses the observed expression levels of five genes and expressed sequence tags (ESTs) involved in the fulfillment and release of bud dormancy. Leida et al. (2012) observed that DAM5 of the EVG gene cluster, and three ESTs are overexpressed in high chill cultivars, while an EST (PpB63) is overexpressed in low-chill cultivars. In summary, DAM genes play a distinct role in growth cessation and maintenance of dormancy release. The expression of DAM genes is also gradually repressed during the winter as buds are exposed to chilling temperatures, allowing growth to resume. Current studies, however, are correlative, and therefore precise functions such as specific binding sites, and protein-protein interactions, are yet to be elucidated.

13.4 Climate Change and Plant Pathogens

Several aspects of plant adaptation in the face of climate change have been explored to a great extent. In particular, the effect of increasing temperature, drought stress, increased precipitation and submergence tolerance (Bailey-Serres et al. 2010) have been the subjects of multiple studies and a few genes have even been identified that confer tolerance to these abiotic stress factors. For instance, in rice, the SUB1 gene, which confers tolerance to submergence, has been introgressed via conventional breeding from the landrace originally found into several high yielding, multiple resistant varieties (Bailey-Serres et al. 2010). Furthermore, there have been some recent reports of evolution due to climate change in natural populations of crop relatives (Nevo et al. 2012). However, there have been comparatively fewer analyses exploring how different scenarios of climate change can affect or benefit plant pathogens (Coakley et al. 1999; Garrett et al. 2006, 2009; Luck et al. 2011) and there is a need for more detailed analyses and constant monitoring.

This lack of studies may be, at least in part, due to the fact that modeling plant responses to abiotic factors is easier than analyzing the effect of those abiotic factors on plant pathogens, and then estimating the outcomes in terms of disease incidence and severity in plants. The multidimensional interaction of variables that determine the onset of plant disease, namely, plant genotype, environmental variables and pathogen genotype, increases the unpredictability of the outcome (Petzoldt and Seaman 2005). Climate change can have an effect on the distribution of individual pathogen species and their virulence. Its effects can also influence the diversity and

composition of the plant pathogen community in a particular geographic region. In addition to climate change, other human activities, such as deforestation, habitat degradation and anthropogenic introduction of parasites can lead to the unprecedented emergence of plant pathogens (Anderson et al. 2004).

Petzoldt and Seaman (2005) predicted that, in temperate regions, warming would lead to an earlier onset of fungal diseases and longer periods of temperatures suitable for pathogen growth and reproduction. For example, in potatoes grown in the Northeastern USA, for each 1 °C of increased temperature, late blight (caused by the oomycete *Phytophthora infestans*) would occur 4–7 days earlier, and the susceptibility period would be extended by 10–20 days. Additionally, pathogens will extend their areas of distribution, both in elevation and latitude, and in some cases they will remain viable through mild winters (Coakley et al. 1999; Petzoldt and Seaman 2005).

Indeed, evidence from many countries suggests that global warming is causing pathogens and other wild species to advance towards the poles at a fast rate (Barford 2013). Some fungi and insects are migrating towards the poles at an average speed of 2.7 km per year, a rate that coincides with the change in median temperature (Bebber et al. 2013). The distributions of many terrestrial organisms are shifting in latitude and elevation, arguably as a result of global warming (Chen et al. 2011). Chen et al. (2011) estimate that several species moved to higher elevations at a rate of 11 m per decade and to higher latitudes at 1.7 km per year. Bebber et al. (2013) and Chen et al. (2011) both found that individual species and taxa vary greatly in their rates of change, but the cause of this variation remains to be determined.

Latitudinal range expansion of crop species relative to their wild species has been common. For instance, rice, a tropical species, is now cultivated more than 10° north and south of the wild species distribution. The same has happened in maize, where Native Americans grew this tropical crop to 45° north and 40° south, and to over 2000 m in elevation. In common beans, *Phaseolus vulgaris*, Bitochi et al. (2012) found strong support for the hypothesis of a Mesoamerican origin of this domesticated species. They observed a high genetic diversity and a marked population structure in Mesoamerican populations, and a drastic reduction in diversity in South American populations. Thus, while an anthropogenic range expansion occurred, this entailed a reduction in diversity perceived in most modern cultivated varieties. Mesoamerican landraces and wild accessions are therefore the most important sources of genetic diversity needed to develop new bean cultivars for adaptation to climate change (Bitocchi et al. 2012), particularly for warmer weather and the pathogens that thrive under those conditions.

Not surprisingly, domesticated plant range expansions have also led to the geographic dispersion of pathogens adapted to these hosts. A dramatic and remarkable case is that of potatoes, which were domesticated by the Incas in the Andes thousands of years ago. Four centuries ago, Europeans took these polyploid tubers and dispersed them around the world, making the potato the world's fourth-largest food crop that is not a cereal. By the 1800s, the potato was a staple for people in Northern Europe, particularly among the poorest farmers. However, in the 1840s, *Phytophthora infestans*, the late blight pathogen reached Europe and caused appalling famines.

Interestingly, this pathogen did not originate in the Andes, but rather in Mexico, another center of diversity for the genus *Solanum* (Goss et al. 2014). Since its dispersal across America, and particularly since its arrival in Europe, *P. infestans* has become the most difficult and expensive pathogen to control, most likely due to the more than 50 different families of effector molecules present in this pathogen's genome. Moreover, another migration event from Mexico to Europe in the 1970s lead to introduction of the A2 type, which can now reproduce sexually with the A1 type and generate new genotypes faster, also leading to the formation of overwintering oospores. Thus, plant range expansion and appearance of pathogens out of their original distribution have happened for a long time. However, there is abundant evidence that both processes are now occurring faster, and due to global transportation of goods, pathogens are being transported rapidly, frequently and over long distances (Anderson et al. 2004; Shaw and Osborne 2011), sometimes leading to the devastation of unadapted plant species.

The adaptive ability of plants, particularly those with long life cycles, may be smaller than that of microorganisms, which, in general, have extremely large effective population sizes (Ne), and although (plant) pathogens tend to have smaller Ne than free-living organisms, they tend to be higher than those of their hosts (Lynch 2006). In fact, microorganisms tend to generate and maintain high standing genetic diversity and are able to adapt rapidly to changing environments. Recent data by Leducq et al. (2014) show that *Saccharomyces paradoxus*, a free-living yeast from temperate regions, has several genetically distinct groups which appear to be locally adapted to distinct climatic conditions. Within-group fitness components showed a correlation to climatic variables. Thus, even a ubiquitous microorganism shows local adaptation and maintains standing genetic variation for climate related traits. While this type of detailed study takes into account climatic variables and microbial diversity, this kind of experiment has not been done for plant pathogenic microorganisms. However, plant pathogens display great diversity, behaving as haplotype clouds, and are able to generate diversity through several mechanisms including somatic mutation, meiosis and parameiosis (Souza-Paccola et al. 2003). Plant pathogens represent an ever-changing environmental factor and due to their constant and strong selective pressure, plants have therefore developed a multitude of mechanisms to become resistant to all but a few potential pathogens in a given environment (Zamora et al. 2009). Indeed, signals of selection have been found at many loci involved in disease response, including balancing selection favoring the generation of new variants through recombination and maintenance of diversity at R genes (Bakker et al. 2006) and the tomato Pto gene (Rose et al. 2007). In *Capsella*, genetic diversity at R genes remained across a population bottleneck, in contrast to reference genes (Gos et al. 2012). Thus, plant evolution is an ongoing process, to a large extent, due to pathogen pressure.

In Israel, wild wheat and barley have adapted to climate change over the last three decades (Nevo et al. 2012). Plants today differ from accessions collected ca. 30 years ago in flowering time and SSR marker allele frequency distribution, suggesting that changes occurred for several phenotypes due to global warming, and this underlines a need to conserve and use genetic diversity from crop species and

their relatives to adapt rapidly. Flowering time is also expected to happen earlier in wheat (Zhang et al. 2004). These researchers used the wheat growth model Sirius and climate data from two regions in China simulated using PRECIS and determined that wheat *Fusarium* ear blight (FEB) incidence would increase substantially under the A1B climate change scenario (Stocker et al. 2013). This suggests rapid breeding wheat for resistance to FEB is necessary to avoid the risk of serious epidemics and substantial reductions in yield in the near future (2020–2050), as is currently the case in maize, with an overall yield reduction of 3.8 % compared to the theoretical maximum (Lobell et al. 2011). However, breeding efforts may be hindered by yet another related anthropogenic effect, viz. extreme genetic diversity depletion and local extinction of wild relatives and landraces due to habitat loss. Ironically, the highly diverse tropical regions that can provide the greatest genetic diversity for crop adaptation to warmer climate, and the pathogens that they carry, are disappearing at the fastest rate due to substitution of diverse cropping systems and their diverse landraces for extensive monocultures. Such monocultures, composed of highly homogeneous and genetically uniform varieties, have a minimal adaptive potential and at the same time represent a bonanza for pathogens as well as a vulnerability for farmers (Zhou et al. 1997).

Additionally, plant breeding techniques aimed at selecting varieties having resistance factors to multiple diseases often involve the simultaneous inoculation of multiple strains of pathogens. Although this has not been demonstrated, simultaneous inoculation practices may give pathogens the upper hand when it comes to generating new genotypes with potentially greater adaptation (Schardl and Craven 2003). For example, interspecific recombination has led to the generation of a new cassava geminivirus (Zhou et al. 1997).

13.4.1 Work on dQTLs and Meta QTL Analyses

The identification of disease resistance or susceptibility related regions in the genome has been an important target ever since the beginning of the development of plant QTL mapping methods. Over the last decade, these studies have shed light on the knowledge of the content, organization and effect of disease QTL (dQTL) in plant genomes and of plant-pathogen interactions. Notably, they have provided valuable knowledge, and, of course, genetic variation and disease-resistance genes, for plant breeders to use in crop improvement. In many cases these resistance genes have been introgressed from rare local landraces or even wild species. Many genes of major effects (R genes), as well as a myriad of dQTLs with smaller effects, that confer resistance against a diverse set of pathogens, have been mapped, and a few have been cloned in many plants, but mostly in the Poaceae and Solanaceae families (Grube et al. 2000; Wisser et al. 2005). Many of those clones, major genes, correspond to canonical R genes containing nucleotide binding site (NBS) and leucine rich repeat (LRR) domains (Martin et al. 2003). Although R genes are typically dominant in inheritance, minor genes often modulate their effects. In fact, R genes or clusters of

R genes have been identified in QTL studies, which suggest a quantitative inheritance more so than a Mendelian inheritance (Balint-Kurti and Johal 2009). Grube et al. (2000) analyzed the conservation of taxonomic specificity and genomic position of R genes in the Solanaceae. They found that R genes, defined phenotypically in previous QTL analyses, occur at corresponding positions in several Solanaceae species. Moreover, while the specificity of these R genes evolves rapidly, their function in initiation of disease response is conserved. Interestingly, only R genes related to resistance against oomycetes have the same position and specificity in different solanaceous crops. This sort of information is highly valuable for transferring information across crops species. Therefore, mapping the results of multiple QTL studies in a single genome, i.e. a meta-QTL analysis, has been a goal in several studies.

In the Poaceae, two analyses of this kind have attempted to summarize the information from all available disease-related QTL mapping studies (Wisser et al. 2005, 2006). Wisser et al. (2005) conducted a meta-QTL analysis in rice and estimates that close to 50 % of the genome is involved in disease resistance. While this could be an overestimation due the large size of the QTL regions, evidence from expression analyses of plants challenged with pathogens suggests that the number of genes involved in microorganism disease response is indeed in the tens of thousands (Pratt et al. 2005; Zamora et al. 2009). R genes are very abundant in plant genomes, rice has ca. 600 (Goff et al. 2002), many are clustered (Bergelson et al. 2001), evolve rapidly under positive selection (Mondragón-Palomino et al. 2002) and are significantly associated with QTL regions in rice (Wisser et al. 2005).

There is great interest in identifying genes or genomic regions involved in broad spectrum, and more durable, quantitative disease resistance. Wisser et al. (2006) mapped over 400 dQTL from 50 different publications in the maize genome and identified clusters of dQTL for multiple diseases. More recently, Kump et al. (2011) used arguably the most advanced mapping population available in maize (and all crop plants) today and found that resistance to southern corn leaf blight is determined by many loci of small and additive effects. Quantitative disease resistance is clearly going to be essential in coping with new pathogens and pathogen's strains under climate change.

One aspect that requires further analysis is the effect of the abiotic environment on disease incidence. In general, disease symptoms are often dependent upon temperature. For instance, in potato, warmer temperatures favor the development of viral disease symptoms, while in cooler temperatures the presence of the virus can go unnoticed (DeBokx and Piron 1977). Wheat and oats show increased susceptibility to biotrophic fungal pathogens with increasing temperature (Coakley et al. 1999). This evidence suggests that heat-stressed plants become more susceptible to pathogens. Thus, pathogens already prevalent in the environment may become a bigger threat to crops and producers as temperature increases. Furthermore, this phenomenon indicates that our ability to correctly diagnose a disease and its potential effect in terms of yield depends on the environmental temperature. Also, there is a possibility that QTLs important for disease resistance under higher temperatures were missed, or were not reported in the literature, precisely because they showed a high GxE, and were not present in all years or test sites.

13.4.2 Genome Content and Structure Under Disease QTL and Plant Capacity for Adaptation to Climate Change

Plants are at the base of the food chain in every ecosystem and, as such, must endure the attack of an innumerable diversity of organisms, including pathogens such as viroids, viruses, phytoplasmas, bacteria, fungi, oomycetes, protozoans and even parasitic angiosperms. They also have to fend off pests such as nematodes, insects, mites and even large mammalian herbivores.

Considering the diversity of potentially pathogenic microorganisms, their large effective population sizes, and therefore, their rapid response to selection, it is intriguing that multicellular organisms, with much smaller effective population size do not succumb completely. Plants in particular, being the primary producers, are constantly attacked by a large array of organisms trying to take advantage of their energy. Although to determine the amount of energy a plant loses to pathogens is difficult, and therefore its reduction in fitness, a global average of 30 % loss has been estimated for agricultural plants due to pathogens (Agrios 2005). However, in some particular cases, yield losses, i.e. selective pressure, can be close to a 100 %, as has happened during severe epiphytotics such as that on potato by the oomycete *Phytophthora infestans*, causing the Irish Famine of 1846–1847; or some localities during the Southern Corn Leaf Blight Epidemic in the US in 1970, caused by the fungal pathogen *Cochliobolus heterostrophus*; or the Bengal Famine of 1943, caused by the rice brown spot epidemic, also caused by a species of *Cochliobolus* (Agrios 2005). While the incidence of a particular disease may vary from year to year, its presence can be assumed and therefore, each plant species is under a strong and relatively constant selective pressure by a diverse array of pathogens.

Because of their sessility and lack of an adaptive immune system, the plant genome must carry numerous disease resistance genes (DRGs) to deal with many different potential pathogens. Up to 20 % of the plant genome may be involved in disease resistance, which corresponds to ca. 5,800 genes in *Arabidopsis thaliana*, and close to 8,000 genes in the rice genome (Goff et al. 2002; Yu et al. 2002), ca. 600 of which are canonical R genes, with nucleotide binding site and leucine rich repeat domains (NBS-LRR). Expressing all of these genes constitutively would not be energetically feasible for a plant, and therefore most of these are expressed as needed. This transcriptional reprogramming occurs rapidly, directed by the integration of information from multiple signal transduction pathways, and has led to the identification of many DRGs by analyzing changes in their expression profile (Pratt et al. 2005).

13.4.3 Strategies Used to Identify Disease Resistance Genes

Several different strategies have been used to find disease resistance genes including map-based cloning; gene tagging with insertional mutagens; candidate gene analysis; bioassays to test for in vitro antimicrobial activity of purified proteins; search

for signal transduction regulators and their overexpression or modification and finally whole genome analysis using genome subtraction and expression profiling. Probes and degenerate primers have been used successfully to clone and sequence resistance gene analogs (RGAs) from several species based on conserved domains in the genes known (McIntyre et al. 2004). Recent comparative genomic strategies involve the comparison of syntenic regions of fully- and partially-sequenced genomes where there is evidence of dominant R genes and QTLs involved in the trait of interest (Mazourek et al. 2009). These types of analyses will undoubtedly facilitate the transference of information across species and speed breeding efforts. For example, Huang et al. (2005) cloned the late blight resistance gene R3a from potato based on I2 from tomato. Nevertheless, this approach is limited to those types of genes already known and that have not diverged significantly.

More recently, reverse genetic strategies have been devised to detect the effect of selection in particular genes or regions of the genome. These analyses can be conducted without an a priori hypothesis of the function of these genes, but it may also be possible to try to enrich the sample of candidate genes studied to include genes having some evidence of being used in disease resistance. One such source of evidence is the expression profile of a particular gene as a result of challenging a plant with a pathogen or some kind of abiotic stress. Traditionally, northern blots have been used to study the expression profile of a single gene under particular circumstances, but more recently, high-throughput methods of analysis such as microarrays and EST-based expression profiles (also known as electronic northern, e-northern) have been used to associate particular genes with functions (Pratt et al. 2005). For instance, Zamora et al. (2009) combined *Sorghum* gene expression data with comparative evolutionary data and identified a set of genes that were both highly expressed under pathogen attack and also divergent with respect to rice. This is consistent with the hypothesis that disease response genes are upregulated when needed, and that some of them evolve faster due to the antagonistic coevolutionary *arms race* that defines the interaction between pathogens and their host plants at the molecular level. Clearly, the annotation of genes of interest should be confirmed by multiple methods, but high throughput schemes allow for the analyses of tens of thousands of genes simultaneously.

13.4.4 Categories of DRGs Identified by Fine Mapping of QTL and Other Strategies

Most of the plant disease resistance genes (R genes) cloned so far have two conserved domains: a nucleotide binding site (NBS) and a carboxy terminal leucine rich repeat (LRR) (Ellis et al. 2000). Two categories of NBS-LRR exist, those that have a toll/interleukin-1-receptor (TIR) homology region and those that do not. Toll is a transmembrane protein of *Drosophila melanogaster* involved in growth regulation and is homologous to the vertebrate interleukin-1 receptor. Collier and Moffett

(2009) and Martin et al. (2003) provide reviews on the structure and function of R genes. At least 1 % of the *Arabidopsis* genome is composed of NBS-LRR type genes (ca. 200) and around 75 % of those genes are TIR-NBS-LLR (Ellis et al. 2000; Grube et al. 2000). R-genes lacking the TIR domain often have another conserved domain, the coiled coil (CC) domain, which is plant specific and has a function analogous to that of TIR (Fluhr 2001).

13.4.5 Mechanisms for Rapid Evolutionary Change and Adaptation to Anthropogenic Climate Change

Although there are landrace-specific genes (NBS-LRR receptor-like kinases) that confer disease resistance to biotrophic pathogens through a hypersensitive response, or even extreme resistance without cell death to viral pathogens (Bhattacharjee et al. 2009), disease resistance is highly polygenic in nature. Plants are thus resistant to most potential pathogens in the environment and susceptible only to a few specialists, which may have lower population sizes than free-living microorganisms (Lynch 2006). Additionally, many genes of small and generally additive effects (Kump et al. 2011), often with many alleles (Rose et al. 2007), produce a great variation in terms of disease phenotype. All of this seems to point to a genetic architecture of disease resistance which is an adaptation in itself, that generates functional diversity and which is selected for resilience and rapid evolution. There are several features in the genome of plants species, particularly the ones that we have selected as crops, e.g. maize and wheat that allow for rapid change and adaptation (Dubcovsky and Dvorak 2007). On the other hand, recent evidence indicates that microorganisms play an essential role in the homeostasis of their host (Arnold et al. 2003; Da et al. 2012; Elbeltagy et al. 2000; Vetrivelkalai et al. 2010). There are a multitude of genes involved in the interaction of plants with beneficial microorganisms and combining genomes in a mutualistic symbiosis has given some plant taxa an evolutionary edge. For instance, the interaction of legumes with nitrogen-fixing rhizobia may be the reason this plant family is so successful in terms of numbers of species and total number of individuals. This is a particularly outstanding benefit in regions with poor soils (Sprent et al. 2013).

Plant breeders have achieved introgression of adaptive traits from wild plants in rice (e.g. SUB1, the rice submarine gene) and many other species. In tomato, superior alleles for size and quality were introgressed from plants that did not show any evidence of these traits (Frary et al. 2000). In rice, wide crosses with *Oryza rufipogon* increased yield of the US variety Jefferson by over 20 % in 3 years, compared to a 1 % yearly increase in conventional breeding programs (McCouch et al. 2007). Additionally, introgression of adaptive traits from wild relatives has probably occurred naturally and spontaneously in all crops since the beginning of agriculture (Elias et al. 2001; McKey et al. 2010), leading in some cases to the development of novel and highly valuable commodities, as in the case of wheat (Dubcovsky and Dvorak 2007).

More recently, researchers have looked at the possibility of simultaneously increasing tolerance to both biotic and abiotic stresses. Plant responses to multiple stress factors is different from that elicited from a single stress, and plant hormones, in particular abscisic acid, play a major role in coordinating actions against multiple threats (Atkinson and Urwin 2012). Since abiotic stress factors can lead to enhanced susceptibility to biotic pathogens, and vice versa, breeding strategies devised to counter the effects of climate change must have as a major goal the combination of qualitative and quantitative tolerance factors to both stress sources. The identification and deployment of common regulators, such as transcription factors, that are at the convergence point of action against both biotic and abiotic stress is a priority (Kissoudis et al. 2014).

13.5 New Approaches and Techniques

QTL analysis is an effective, genome-wide approach for detecting marker regions significantly associated with a trait of interest; however, the method is time consuming. First, one must cross two genetically distinct individual plants to generate a mapping population that segregates for the trait or traits of interest. Second, the population is saturated with molecular markers to construct a genetic linkage map. Third, the population is phenotypically evaluated for multiple years and analyzed for significant QTLs. Although the method is effective at locating associated LG segments, the significant regions can still encompass a large range of markers. To further zoom in on QTLs of interest, fine mapping or high-resolution mapping is used to locate closely linked molecular markers (Collard et al. 2005). Although there is no standardized or set number of individuals in a segregating population for fine mapping, analyzing a large population, made up of 1000 individuals or more, can achieve a resolution of <1 cM between markers (Collard et al. 2005; Li et al. 2003). This represents a significant amount of time, and resources. Therefore, alternative and complementary approaches should be explored, such as robust analyses that identify QTLs without the need of a biparental mapping population, alternative population designs, fast, reliable phenotyping methods for large sample sizes and shortened plant life cycles. Additional approaches not only augment QTL and similar genetic studies, but also offer alternatives to crop improvement under global climate change.

13.5.1 Genome Wide Association Mapping and Genomic Selection

Genome wide association mapping (GWAS) identifies significant QTL regions without the need of generating a mapping population. This curtails the task of having to select two contrasting parents, cross them, generate the seed, as well as

germinate and plant the population. Additionally, there is the task of constructing the genetic linkage map, which carries the uncertainty of sufficient marker coverage. Contrary to the traditional approach, GWAS uses the already existing SNPs in the genome and compares the percentages in nucleotide types between the two contrasting traits. Thus, this can relate a SNP to the phenotype of interest. This is exceedingly advantageous for perennial crops, since populations require several years for maturity before they can be evaluated.

Currently, there are two types of GWAS, a population-based approach and a family-based approach (Mitchell-Olds 2010). In population-based GWAS, populations of unrelated individuals are used to examine associations between SNPs and phenotypes. In family-based GWAS, pedigrees derived from crosses among different founding genotypes are used instead. Population-based GWAS takes advantage of the historical recombination events that have accumulated over time, with the disadvantage of finding false positives. Family-based GWAS can eliminate false-positives that occur in the population-based approach because of population structure, but recognizes fewer recombination events. Some studies have already applied the population-based approach. Atwell et al. (2010) have mapped QTLs related to 107 phenotypes in *Arabidopsis*, including flowering, cold tolerance, salt tolerance and pathogen resistance of 200 *Arabidopsis* inbred lines with more than 200,000 SNPs. The family-based GWAS approach has been applied in maize, where a nested association mapping (NAM) population was developed to characterize flowering regulation (Buckler et al. 2009; McMullen et al. 2009). The NAM population consists of 25 groups of inbred lines, each group consisting of 200 inbred lines derived from 25 parents crossed to a sequenced genotype (B73). In other crop species however, complex family structures, such as NAM, is a large economic investment, and the necessary experimental conditions can be difficult to obtain. Another consideration is the availability of defined plant collections in particular crop species. Therefore, GWAS offers important alternative or complementary approaches to traditional QTL analysis.

Breeding for complex traits by marker-assisted selection (MAS) is possible if the trait is explained by major or large effect QTLs, however this is less effective for traits that have multiple QTL regions with small effects (Dekkers and Hospital 2002). In conjunction with GWAS, genomic selection (GS) can be used for polygenic traits, in place of traditional MAS. MAS uses a few markers to explain the total genetic variance, whereas GS predicts breeding values by analyzing the individual's phenotypes and high-density marker scores (Meuwissen et al. 2001). GS addresses the deficiencies of MAS by incorporating all marker information in the prediction model and capturing more of the variation due to minor QTLs (Heffner et al. 2009). Being able to avoid biased marker effects can be useful when breeding for quantitative traits composed of several regions with minor effects (minor QTLs) or when pyramiding several resistance genes.

13.5.2 Nanotechnology and Biosensors

Identification of significant QTLs requires phenotyping numerous individuals within study populations plus multi-year evaluations. Although current genotyping technology can process large sample sizes and generate vast amounts of data, phenotyping large populations for complex traits is still a limitation. High-throughput phenotyping methods in the context of quantitative traits such as heat tolerance, or nutrient and carbohydrate composition, can accelerate QTL discovery. New technological advances, however, can aid in developing faster, high-throughput phenotyping tools. Nanoparticle and nanotechnology is an area of research that has gained overwhelming recognition and has expanded to many fields and applications, including clinical diagnosis, environmental monitoring and food analysis (Fanali et al. 2011; Sapsford et al. 2013; Sattler 2011; Suginta et al. 2013; Wu et al. 2007; Zhang et al. 2003). An area of particular interest is the development of biosensors, carbon nanotubes or metal nanoparticles in suspension linked to a specific probe that quantifies molecules of interest (Sattler 2011). Often the probes are bioactive molecules that retain their function when adsorbed by or are immobilized onto a nanostructure (Luo et al. 2006). These probes range from enzymes such as horseradish peroxidases (Xiao et al. 1999), to oligonucleotide probes that function as electrochemical DNA sensors (Bai et al. 2006). Although these are useful for numerous applications, biosensors have not been extensively used (if at all) for phenotyping breeding populations, nor large plant populations in QTL analyses. One example where biosensor technology could aid the field of phenomics and QTL discovery is by the use of quantum dots (QD) for quantifying phenolic compounds and other important molecules. QD are colloidal nanocrystalline semiconductors possessing unique spectral properties (Akshath et al. 2014). These structures are used as enzymatic biosensors because of the electron transfer that modulates its fluorescent properties, i.e. quenching of the QDs' fluorescence (Clarke et al. 2006). Akshath et al. (2014) have successfully used immobilized laccase-QD technology to convert polyphenols into quinones as an ultrasensitive optical label to detect polyphenols in 15-min assays. Other potential applications include antibody bound nanoparticles that can detect proteins of interest, such defense response proteins or viruses in plants. Huang et al. (2012) developed a modified graphene paste electrode with gold nanoparticles and a Nafion-L-cysteine composite film as an electrochemical immunosensor for the detection of hepatitis B surface antigen. Liu et al. (2013) developed an electrochemical immunosensor for ochratoxin A by combining a polythionine/gold nanoparticle composite film and an enzyme catalysis amplification. Further advancements in this technology allow us to envisage QD assays, nanoparticle devices, or nanotube sensors that can detect molecules of interest in a relatively short time, and in multi-well assays to evaluate large mapping populations. The range of molecules can be vast, from resistance gene-products, to volatiles such as aroma and flavor compounds.

13.5.3 Fast-Track Breeding

One of the most limiting factors for breeding woody perennial crops is the long juvenile period. In tree species, time to maturity can take 5–40 years or more (Flachowsky et al. 2009); poplar trees can take 7–10 years and citrus trees can take up to 20 years to flower (Hsu et al. 2006; Peña et al. 2001). In contrast to annuals, applying basic breeding methods in woody perennial species, such as backcrosses, or mass selection, can amount to decades and an exorbitant economic investment over time. Introgression of a single gene through backcrossing to a recurrent parent is next to impossible under these circumstances; starting at the seedling stage, two backcrosses in citrus crops would take 40 years. Breaking or shortening the juvenile period is of high priority in tree crops and woody perennials, and there is extensive research on the subject, however this has been generally accomplished through cultural practices, such as grafting, or through chemical or physical methods (Fischer 1994; Hanke et al. 2007; Meilan 1997). Breeding for shorter juvenile periods by conventional means is possible; however, this is not feasible as natural, early-flowering mutants are rare in woody plants (Flachowsky et al. 2009). On the other hand, transformation approaches are demonstrating high potential. Recent experiments show that the juvenile period can be significantly shortened when trees are transformed with flower-inducing genes, such as FLOWERING LOCUS T (FT) and MADS4, controlled by constitutive or inducible promoters (Flachowsky et al. 2007, 2009; Wenzel et al. 2013). Conversely, flower-inhibiting genes, such as TERMINAL FLOWER 1 (TFL), are silenced to induce precocious flowering (Freiman et al. 2012). Both FT and TFL directly affect flowering, but genes that indirectly promote flowering, either by overexpression or inactivation, can too lead to shorter juvenile periods, such as the CONSTANS (CO) gene; inactivated CO causes late flowering while its overexpression induces early flowering (Putterill et al. 2004; Wellmer et al. 2006).

This approach has been successful in several tree crops. Overexpression of the birch (*Betula pendula*) BpMADS4 gene, a homolog of FRUITFULL (FUL), dramatically reduces flowering time in birch and apple (Elo et al. 2007; Flachowsky et al. 2007). Constitutive expression of the *Arabidopsis* non-MADS-box meristem identity gene, LEAFY (LFY), induces early flowering in transgenic poplar (*Populus*) and *Citrus* plants (Peña et al. 2001; Rottmann et al. 2000). Constitutive expression of the poplar FT homologue, FT2, induces early flowering with high efficiency (Hsu et al. 2006). Similarly, constitutive expression of the poplar homologue FT1 in transgenic plum (*Prunus domestica*) results in continuous flowering, but also alters architecture and dormancy requirements (Srinivasan et al. 2012). Constitutive expression has not been the only approach for shortening juvenile phases. Antisense expression of the apple TFL1-like gene, MdTFL1, silences the expression of endogenous MdTFL1; this promotes early flowering because TFL functions as a floral suppressor (Kotoda et al. 2006). Kotoda et al. (2006) reduced flowering time to 8 months in transgenic-MdTFL1 apple plants, compared to 69 months in non-transgenic controls. The juvenile phase in European pear (*Pyrus communis*) can last

up to 14 years (Freiman et al. 2012; Visser 1964). Freiman et al. (2012) demonstrated that antisense MdTFL1 reduces the expression of both endogenous PcTFL-1 and PcTFL-2 genes in European pear by RNAi-mediated silencing; the transformed plants developed flowers in vitro and within 8 months in greenhouse conditions.

These transgenic studies have paved the way to an innovative, faster approach to developing new woody plant cultivars. Recent studies are now focusing on strategies to incorporate the technology into breeding practices, in very clever ways. Wenzel et al. (2013) transformed the apple cultivar Pinova with the PtFT1 and PtFT2 genes from poplar (*Populus trichocarpa*) driven by the heat-inducible Gmhsp 17.5-E gene promoter from soybean (*Glycine max*) to induce flowering. The transgenic apple lines were also micrografted onto Golden Delicious seedlings as rootstocks to examine the transferability of the PtFT1 mRNA through the micrograft junction. Transgenic lines flowered over a period of 28 days upon heat treatment (at 42 °C) and flower morphology and pollen vitality was normal. Although the transfer of the mRNA transcript occurred in only one of the non-transformed scions, the study demonstrates a potential system where early flowering can be induced without the hassle of generating transgenic plants or going through the regulatory process of releasing a transgenic line. Yamagishi et al. (2014) developed a novel technology to induce early flowering into a breeding program. By using the apple latent spherical virus (ALSV), the authors simultaneously overexpressed *Arabidopsis* AtFT and silenced apple MdTFL1-1 genes to promote early flowering in apple seedlings. Virus vector systems can be advantageous because these systems do not alter the host genome and are methodologically simple (Purkayastha and Dasgupta 2009). When apple cotyledons are inoculated with the virus vector (with a 90 % success rate), seedlings can flower within 3 months (Yamagishi et al. 2010). Additionally, ALSV was not transmitted via seeds to successive progenies in most cases. Thus, these new systems could revolutionize breeding programs, and allow tree crops and other crops with long juvenile periods to accelerate cultivar release, and apply breeding methods that are generally limited to annual crops. There is also the added advantage of not having to release transgenic lines with its associated regulation, nor having to transform selections to induce early flowering. Accelerating the breeding process is not only advantageous for generating QTL study populations, but also in the introgression of complex traits as the changing environment will demand faster cultivar release.

13.6 Conclusions and Prospects

An important question to ask is will methods currently available for QTL analysis be able to provide the necessary information to keep up with the overall climate changes? Perhaps this is more a concern in crops with long juvenile periods, where breeding, even with selection tools, takes dozens of years. If the trends in warmer temperatures continue, there will be yield loss due to insufficient chilling accumulation. In Australia there are already predictions that chilling will reach only half of

the expected units by 2030 in Australia's peach growing regions (Topp et al. 2008). This implies that new, low-chill cultivars would have to be released within 14 years or less to allow replacement of current higher chilling cultivars. Similar considerations can be said of other traits such as HT and pathogen resistance. Nevertheless, QTL analysis is an effective method for identifying genes related to stress tolerances and disease resistance. Accurate, fast and cheap, screening methods for complex traits should also be a priority. This widens the option for the application of different techniques in parental selection; in some cases, genotyping methods can be expensive. With the application of new, up-and-coming technology alongside QTL techniques, breeding could take a new leap in accelerating the process. Successful and faster introgression techniques are key in developing superior cultivars adapted to the present and future environments. Incorporating the technological tools into a breeding program will mean successful production under new climatic conditions.

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Chapter 14
Genotype x Environment Interaction Implication: A Case Study of Durum Wheat Breeding in Iran

Reza Mohammadi and Ahmed Amri

Abstract The genotype x environment (GE) interaction is a major challenge to plant breeders as it complicates testing and selection of superior genotypes and consequently reduces gains from selection. This chapter introduces and compares different statistical models to handle GE interaction by applying them to the durum wheat breeding program in Iran as an example. The results indicate significant crossover GE interaction suggesting the need for applying appropriate analysis for the exploitation and/or the minimization of GE interaction in multi-environment trials (MET) data. The test locations differed in their discriminative ability and representativeness. Highly significant correlations were found between univariate and multivariate statistical models in ranking genotypes for stability and for integrating yield with stability performances, indicating that they can be used interchangeably. Evaluation of genotypes based on multiple traits data identified parental germplasm for earliness, short stature, high grain weight and high grain yield. The proposed statistical analysis can assist in increasing the efficiency of breeding program through (a) selection of the most discriminate locations, (b) identifying superior genotypes based on both strategies dealing with exploitation and minimization of GE interaction and (c) exploring significant genetic gains in yield and yield stability.

Keywords Durum wheat • GE interaction • Genetic gain • Genotype ranking • Statistical models

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14.1 Introduction

The knowledge of genotype x environment (GE) interaction is of major importance to plant breeders for developing crop cultivars adapted to unpredictable environments. If relative performances and rankings of genotypes grown in different environments are highly different, then GE interaction becomes a major challenging factor to crop breeding programs (Allard and Bradshaw 1964; Zobel and Talbert 1984). GE interactions can be grouped into three broad categories: no GE interaction, non-crossover (quantitative) interaction and crossover (qualitative) interaction. When the number of environments and the number of genotypes increase, the number of possible GE interactions also increases. With only two genotypes and two environments, and with only a single criterion, at least four different types of interactions are possible. Thus, with 10 genotypes and 10 environments, 400 types of interactions are possible, which would certainly make their implications and interpretation more difficult to comprehend. The interactions are usually higher for traits with lower heritability (Allard and Bradshaw 1964). The differential and nonstable response of genotypes to diverse environments is referred to as a crossover interaction when the ranks of genotype change or switch from one environment to another. Crossover interaction implies that no genotype is superior in multiple environments (Muir et al. 1992).

When GE interaction is significant, researchers working on different or unpredictable environments can either choose to develop germplasm specifically adapted to each environment and/or target the selection of genotypes that can generally perform well across many environments (Isik and Kleinschmit 2005). Therefore, it is necessary to assess the environmental sensitivity of genotypes in terms of higher yields and stability. However, ensuring continuous yield gains remains the main objective of crop-breeding programs (Fischer 2007; Reynolds et al. 2009). This can be achieved by increasing the yield potential, which could concomitantly increase yield under mild and moderate stress (Blum 2005; Slafer and Araus 2007), or by improving yield stability (Pswarayi et al. 2008). In many crops and certainly in durum wheat, insufficient yield stability has been recognized as one of the main factors responsible for the gap between yield potential and actual yield, particularly in drought-prone environments (Cattivelli et al. 2008; Tollenaar and Lee 2002). If GE interaction is significant, breeders need to know about genotypes with good performance across a range of environments. Stability may be static or dynamic. Stability is static when the genotype tends to maintain consistent yield across environments and it is dynamic when a genotype performance responds in a consistent fashion to changes in the environment (Becker and Leon 1988).

There are two possible strategies for plant breeders to minimize the effects of GE interaction, namely (i) the subdivision of the heterogeneous target area into more environments with more homogeneous conditions and to breed for each specific environment and (ii) the selection of varieties which show a high degree of stability in performance over a wide range of environments (Tai 1971). However, the first strategy can still encounter large interactions of genotypes with locations even

within one site (sub-region) due to unpredictable conditions from season to season. Consequently, breeders will always be faced with significant GE interactions which will complicate the identification of superior genotypes. The interpretation of GE interactions can be facilitated by the use of several statistical modeling methods. These models can use linear joint-regression (Becker and Leon 1988; Eberhart and Russell 1966; Finlay and Wilkinson 1963; Tai 1971; Yates and Cochran 1938); multivariate clustering techniques (Lin and Butler 1990); multiplication approaches such as additive mean effects and multiplicative interaction (AMMI) (Gauch 1992; Zobel et al. 1988) and genotype plus GE (GGE) biplot analysis (Yan et al. 2000). Modeling GE interaction in multi-environment trials (METs) helps to determine phenotypic stability of genotypes, but this concept has been defined in different ways and a large number of stability parameters have been developed (Gauch and Zobel 1996).

A number of parametric (univariate and multivariate) and non-parametric stability procedures have been developed over the years to analyze GE interaction especially yield stability over environments. Huehn (1996) indicated that there are two major approaches for studying GE interaction to assess the stability/adaptability of genotypes. The first and most commonly used approach is parametric, which relies on distributional assumptions about genotypic, environmental and GE interaction effects. The second is the non-parametric approach, which does not require any assumptions. Non-parametric stability procedures proposed by Huehn (1979), Nassar and Huehn (1987), Kang (1988) and Fox et al. (1990) are based on the ranks of genotypes in each environment, and the genotypes with similar ranking across environments are classified as stable.

Among parametric stability measures, the linear regression of genotype values on environmental mean yield (Finlay and Wilkinson 1963; Yates and Cochran 1938), frequently termed *joint regression analysis*, is undoubtedly the most popular (Becker and Leon 1988; Romagosa and Fox 1993) due to its simplicity and the fact that its information on adaptive responses is easily applicable to locations other than the chosen test sites. Finlay and Wilkinson (1963) considered linear regression slopes as a measure of stability. Eberhart and Russell (1966) emphasized the need of considering both the linear (b) and non-linear (S^2di) components of GE interactions in judging the stability of a genotype. Breese (1969), Samuel et al. (1970) and Paroda and Hayes (1971) emphasized that linear regression could simply be regarded as a measure of response of a particular genotype, whereas the S^2di is the most suitable measure of stability. Shukla (1972) presented a statistic called *stability variance* (σ^2), which is identical to the Wricke (1962) ecovalence in the ranking of genotypes for stability (Kang et al. 1987). Some other univariate stability parameters are: the Tai (1971) environmental effects (α) and deviation from the linear response (λ), superiority index (Pi) (Lin and Binns 1988), environmental variance (S^2x) (Roemer 1917, as reported in Becker and Leon 1988), etc. All these methods are parametric.

Non-parametric stability methods are not generally affected by data distribution and are based on rankings of genotypes. Non-parametric methods have some advantages over parametric stability methods in allowing the reduction of the bias caused

by outliers and no assumptions are needed about the distribution of observed values. They are easy to use and to interpret, and additions or deletions of one or a few genotypes have little effect on the results (Huehn 1990). Furthermore, if the breeder is only interested in the existence of rank order differences over different environments, the non-parametric statistics for GE interactions based on ranks provide a useful alternative to parametric approaches currently used. In these cases, the relative characteristics and comparisons of the genotypes are more important than absolute characterization and comparisons. As these methods are based on ranks and not on values, a genotype is considered stable if its ranking is relatively constant across environments (Flores et al. 1998). Several non-parametric methods have been developed to interpret the responses of genotypes to environmental variation (Huehn 1979; Fox et al. 1990; Kang 1988). Huehn (1979) and Nassar and Huehn (1987) proposed four non-parametric measures of phenotypic stability (i) $Si^{(1)}$ is the mean of the absolute rank differences of a genotype over n environments, (ii) $Si^{(2)}$ is the variance among the ranks over the n environments, (iii) $Si^{(3)}$ and (iv) $Si^{(6)}$ corresponding to the sum of the absolute deviations and sum of squares of ranks for each genotype relative to the mean of ranks, respectively. Kang (1988) assigned ranks for mean yield, with the genotype with the highest yield receiving the rank of 1, and the ranks for the stability variance of Shukla (1972), with the lowest estimated value receiving the rank of 1. The sum of these two ranks provides a final index, in which the genotype with the lowest rank-sum (RS) is regarded as the most desirable. Fox et al. (1990) suggested a non-parametric superiority measure for general adaptability. They used stratified ranking of the genotypes and ranking was done at each environment separately: the proportion of environments at which the genotype occurred in the top, middle and bottom third of the ranks was computed to form the non-parametric measures TOP, MID and LOW, respectively.

Among the multivariate models, the additive mean effects and multiplicative interaction (AMMI) (Gauch 1992; Zobel et al. 1988) and genotype (G) main effect plus GE interaction (GGE) (Yan et al. 2000) biplot analysis are the most known and appealing methods for analyzing GE interaction in MET data. These models have been developed and applied over the years to analyze GE interaction and especially yield stability over environments for different crops (Annicchiarico et al. 2005; Gauch 1992, 2006; Suadric et al. 2006; Wamatu and Thomas 2002; Yan et al. 2000).

The AMMI model is a hybrid analysis that incorporates both the additive and multiplicative components of the two-way data structure (Shafii and Price 1998; Shafii et al. 1992). The AMMI biplot analysis is considered to be an effective tool to diagnose the GE interaction patterns graphically. There are several possible AMMI models characterized by a number of IPC axes ranging, for g genotypes and e environments, from zero (AMMI-0, i.e. additive model) to a minimum between $(g - 1)$ and $(e - 1)$. The full model (AMMI-F), with the highest number of IPC axes, provides a perfect fit between expected and observed data. Models including 1 (AMMI-1) or 2 (AMMI-2) IPC axes are frequently appropriate in the presence of significant GE interaction. For the AMMI-2 model, the scaled scores of genotypes and locations in the space of IPC1 and IPC2 may be reported in a single graph

(biplot) to appreciate site or genotype similarity for GE interaction effects, and graphically estimate these effects (Annicchiarico 2002).

Yan et al. (2000) developed the GGE biplot methodology for graphical analysis of METs data. The GGE biplot is constructed by plotting the first two principal components (PC1 and PC2) derived from singular value decomposition (SVD) of the environment-centered data. GGE biplot can visually address many questions relative to genotype and test environment evaluation. Increasingly, plant breeders and agronomists have found GGE biplots useful in mega-environment analysis (Casanoves et al. 2005; Dardanellia et al. 2006; Samonte et al. 2005; Yan and Rajcan 2002; Yan and Tinker 2005; Yan et al. 2000), genotype evaluation (Bhan et al. 2005; Fan et al. 2007; Kang et al. 2006; Malvar et al. 2005; Sandhu et al. 2014; Voltas et al. 2005), test-environment evaluation (Blanche and Myers 2006; Dimitrios et al. 2008; Thomason and Phillips 2006; Yan and Rajcan 2002), trait-association and trait-profile analyses (Morris et al. 2004; Ober et al. 2005; Yan and Rajcan 2002). By applying the GGE biplot, genotypes can be evaluated for their performance in individual environment and also across environments, for their mean performance and stability, and for their general or specific adaptations. Simultaneously, environments can be visually evaluated and grouped on the basis of their ability to discriminate among genotypes and their representativeness of other test environments. In addition, a GGE biplot can reveal the *which-won-where* pattern of the MET data, which is important for mega-environment identification and for recommending genotypes specific to each mega-environment (Yan and Tinker 2005). A mega-environment is defined as a group of environments that consistently share the same best genotype(s) (Yan and Kang 2003; Yan et al. 2000).

Trait profiles of genotypes and trait relations are frequently influenced by unpredictable environmental conditions as experienced particularly in Mediterranean rainfed regions. Under these situations, genotype selection should be based on multiple traits evaluated under variable environments within the target region (Mohammadi and Amri 2011). The genotype-by-trait (GT) biplot proposed by Yan and Rajcan (2002) is a statistical tool used for evaluating genotypes based on multiple traits and for identifying those that are superior for desired traits and hence could be candidates for use as parental germplasm in a breeding program or even proposed for commercial release. The GT biplot analysis allows the visualization of genetic correlation among traits (Lee et al. 2002; Ma et al. 2004; Rubio et al. 2004; Yan and Frégeau-Reid 2008; Yan and Rajcan 2002). It has been exploited in variety evaluation of soybean (Yan and Rajcan 2002), white lupin (Rubio et al. 2004), bean (Gonzalez et al. 2006), wheat (Morris et al. 2004), sugar beet (Ober et al. 2005) and oats (Peterson et al. 2005; Yan and Frégeau-Reid 2008; Yan et al. 2007). It also provides information on the usefulness of cultivars for production and helps in detecting less important (redundant) traits, and in identifying traits that are appropriate for indirect selection for a target trait. However, there are pitfalls in interpreting a GT biplot when the biplot does not fully approximate the data (Yan and Frégeau-Reid 2008).

The main objective of this chapter is to introduce a package of statistical models, ranging from univariate to multivariate, to deal with GE interaction in MET data.

Iran regional durum wheat yield trials served as an example to illustrate different approaches. Other specific objectives of this book chapter, in detail, were to: (a) compare the effectiveness of different statistical methods in ranking genotypes in durum wheat using MET data, (b) identify stable and high-yielding durum breeding lines with specific or broad adaptation to rainfed areas of Iran, (c) examine the existing mega-environments within the durum crop breeding program of Iran and (d) identify the trait profiles of tested materials and trait relations.

14.2 Experimental Data and Analyses

14.2.1 Experimental Data

Fourteen genotypes (G1–G14) including 11 durum wheat breeding lines (G1–G11), selected from the durum wheat joint project of Iran/ICARDA, and 3 control varieties were evaluated in 16 environments (combinations of year-location). Entries 12, 13 and 14 were the controls: entry 12 (G12) is the newly released durum wheat cultivar (Saji), entry 13 (G13) is an old durum wheat variety (Zardak) grown in limited area and entry 14 (G14) is a bread wheat landrace (Sardari), which is an outstanding landmark variety grown on a large scale in rainfed cold and moderate cold regions of Iran for over 40 years. The modern cultivar (Saji) is an outstanding durum wheat cultivar, recently released by the Dryland Agricultural Research Institute (DARI), for rainfed and supplemental irrigation conditions in moderate cold and warm regions of Iran, and is well appreciated by farmers. It is a high-yielding cultivar with stable performance, high pasta quality, and resistance to lodging and to the major pests and diseases. These three controls are usually used in the DARI durum breeding program. The 14 entries were evaluated during 4 consecutive cropping seasons (2005–2009) in three research stations representing major durum rainfed growing areas in Iran. The sites included the experiment stations of Kermanshah (moderate cold winter location; 34°19′ N; 47°17′ E, 1351 m AMSL), Ilam (warm location; 33° 41′N; 46° 35′E, 975 m AMSL) and Shirvan (cold winter location; 37°14′N; 58°07′E, 1131 m AMSL). At the moderate and warm locations, the trials were also conducted under supplemental irrigation (one or two irrigations with 25 mm each applied either at flowering and/or at grain filling stages to cope with terminal drought stress which is a common feature in western parts of Iran). However, due to severe drought conditions in the 2007–2008 cropping season, no data were recorded at warm and cold locations but in the moderate location and to avoid crop failure an irrigation of 30 mm before flowering stage was applied for both rainfed and irrigated trials. For all 16 environments, the experimental layout was a randomized complete block design with 3 replications. Plots size was 7.2 m^2 (6 rows, 6 m long and 20-cm row spacing). Weeds were controlled manually as needed. Fertilizer rate was 50 kg N ha^{-1} and 50 kg P_2O_5 ha^{-1} applied at planting. The grain yield (YLD) was recorded for each genotype in all

16 environments. The yield plots were converted to productivity per hectare (kg/ha^{-1}) and subjected to data analysis.

In addition to YLD, several important traits were also recorded for each genotype under both rainfed and irrigated conditions during two cropping seasons (2005–2006 and 2006–2007) at moderate cold location. The traits included plant height (PH), peduncle length (PL), flag-leaf length (FL), spike length (SL), days to heading (DH), days to maturity (DM), biomass yield (Bio), harvest index (HI) and thousand-kernel weight (TKW). Days to heading was designated as the day until 50 % of the plants in the plot had at least one open flower. Days to maturity were when 50 % of the plants in the plot had yellow heads. Plant heights (PH), peduncle length (PL), flag-leaf length (FL) and spike length (SL) were measured for each genotype at physiological maturity stage. The biomass yield (Bio), grain yield (YLD) and harvest index (HI) were also measured from 1 m long in each plot. The TKW was measured for 1000-grains for each genotype.

14.2.2 Data Analysis

14.2.2.1 Univariate Parametric Models

Analysis of Variance The grain yield data of 14 genotypes grown across 16 environments were subject to combined analysis of variance to partition yield variation into environments, genotypes, and GE interaction effects. In MET with *m* genotype and *n* environments, the combined ANOVA of MET data is based on the following equation:

$$X_{ijk} = \mu + G_i + E_j + (GE)_{ij} + b_{jk} + e_{ijk}$$

where X_{ijk} is the phenotypic value of the *i*th genotype in the *k*th replicate in the *j*th environment; μ is mean of all genotypes over all environments; G_i is the effect of the *i*th genotype, i = 1, 2, … m; *E*j is the effect of the *j*th environment, j = 1, 2 ...., n; $(GE)_{ij}$ is effect of the interaction between *i*th genotype and the *j*th environment, b_{jk} is the effect of the *k*th replicate in the *j*th environment, k = l, 2 ..... p; and e_{ijk} is random error deviate on the *i*th genotype in the *k*th replicate in the *j*th environment.

Joint Regression Analysis This model was developed by Yates and Cochran (1938) and offered again, in slightly different forms, by Finlay and Wilkinson (1963), Eberhart and Russell (1966), and Perkins and Jinks (1968). The performance of each genotype in each environment was regressed over the means of all genotypes at each environment. A genotype with a regression coefficient (b) equal to unity and variance in regression deviation (S^2di) equal to zero will be highly stable. The joint regression analysis (JRA) model is as follow:

$$Y_{ij} = \mu + G_i + E_j + b_i E_j + d_{ij} + e_{ij}$$

where Y_{ij} is the mean yield for the *i*th genotype in the *j*th environment; μ is the grand mean; G_i is the effect of genotype *i* (*i* = 1, 2, …, *g*) and *E*j is the effect of environment *j* (*j* = 1, 2, …, *e*); *b*i is the linear regression coefficient of the *i*th genotype on environmental index; *d*ij is deviation from regression; and e_{ij} is the average of the random errors associated with the *i*th genotype and *j*th environment.

The method used by Finlay and Wilkinson (1963) estimated the regression coefficient (*b*) to measure the stability and relative adaptability. Eberhart and Russell (1966) generalized this concept by calculating the deviations (S^2di) from linear regression. According to the Eberhart and Russell (1966) model, the $b = 1$ coupled with low S^2di indicate average stability. When this is associated with high mean yield, genotypes have general adaptability and when associated with low mean yield, genotypes are poorly adapted to all environments. The $b > 1$ describes genotypes with higher sensitivity to environmental change and greater specificity of adaptability to high yielding environments, while the $b < 1$ provides a measurement of greater resistance to environmental change, and therefore increasing the specificity of adaptability to low-yielding environments.

The Tai Stability Analysis In the Tai (1971) stability analysis, the interaction term is partitioned into two components: the linear response to environmental effects, which is measured by a statistic (α), and the deviation from the linear response, which is measured by another statistic (λ). A perfectly stable genotype has (α, λ) = (−1, 1) and a variety with average stability is expected to have (α, λ) = (0, 1). Tai's analysis also provides a method of obtaining the prediction interval for $\alpha = 0$ and a confidence interval for λ values, so that the genotypes can be distributed graphically in different stability regions of the Tai plot. The Tai (1971) stability statistics (α and λ) for each genotype separately and the Tai plot were estimated by the SAS program developed by Fernandez (2000).

Other Stability Methods Several other stability parameters were used for the assessment of GE interaction in MET data.

Environmental variance (S^2x) of genotypes detects all deviations from the mean (Roemer 1917, cited in Becker and Leon 1988). A genotype with minimum variance under different environments was considered to be stable. Superiority index (Pi) was defined as the distance mean square between the genotype's response and the maximum response over environments (Lin and Binns 1988). A low value of Pi indicates high relative stability. An unbiased estimate using stability variance (σ_i^2) of genotypes was determined according to Shukla (1972). Geometric adaptability index (GAI) (Mohammadi and Amri 2008) was calculated in which the genotypes of greatest interest would be those with the highest GAI-values and consequently could be regarded as widely adapted.

14.2.2.2 Univariate Non-parametric Models

The four non-parametric measures of phenotypic stability proposed by Huehn (1979) and Nassar and Huehn (1987) were used in stability analysis: (1) $S_i^{(1)}$ is the mean of the absolute rank differences of a genotype over n environments; (2) $S_i^{(2)}$ is

the variance among the ranks over the n environments; (3) $S_i^{(3)}$ and (4) $S_i^{(6)}$ are the sum of the absolute deviations and sum of squares of ranks for each genotype relative to the mean of ranks, respectively. The genotypes with low values for these parameters can be regarded as stable.

The Kang (1988) rank-sum (RS) is another non-parametric stability measure which uses both yield and the Shukla (1972) stability variance. This index assigns a weight of one to both yield and stability statistic to identify high-yielding and stable genotypes. The genotype with the highest yield is given a rank of 1 and a genotype with the lowest stability variance is assigned a rank of 1. All genotypes were ranked in this manner, and these ranks are added for each genotype. The genotype with the lowest RS is the most desirable one.

The stratified ranking technique of Fox et al. (1990) consists of scoring the number of environments in which each genotype ranked in the top, middle and bottom thirds of trial entries. The proportion of environments at which the genotype occurred in the top, middle and bottom third of the ranks was computed to form the non-parametric measures of TOP, MID and LOW, respectively. A genotype that occurred mostly in the top third (high value of TOP) was considered to be a widely adapted genotype.

The yield stability (YSi) statistic was generated as outlined by Kang (1993). Ranks were assigned for mean yield, with the genotype with the highest yield given a rank of *l* (*l*=number of genotypes, which here is equal to 14). Similarly, ranks were assigned for the stability parameter with the lowest estimated value receiving the rank of 1. Stability ratings were computed as follows: −8, −4, and −2 for stability measures significant at $P<0.01$, 0.05, and 0.1, respectively; and 0 for the non-significant stability measure. The stability ratings of −8, −4, and −2 were chosen because they changed genotype ranks from those based on the yield alone. The YSi statistic, which is an integrated measure of yield and stability of genotypes, evaluated in METs would help in identifying the genotypes with high and relatively stable yields (Kang 1993).

14.2.2.3 Multivariate Statistical Models

AMMI Model The GE interaction was analyzed through the additive main effect and multiplicative interaction (AMMI) analysis (Gauch 1992) using IRRISTAT software (IRRI 2005). The AMMI results were graphically presented in the form of a biplot (Gabriel 1971), where genotypes and environment scores of the first two bi-linear terms are represented by vectors, with their starting points at the origin (0, 0) and end points (markers) determined by their scores (Crossa 1990; Gauch and Zobel 1988; Zobel et al. 1988). The AMMI model is as follow:

$$\mathrm{Yge} = \mu + \mathrm{G_i} + \mathrm{E_j} + \sum_{k=1}^{n} \lambda_k \gamma_{ik} \delta_{jk} + \rho_{ij} + \varepsilon_{ijk}$$

where Yge is the yield of genotype G in environment E; μ is the grand mean; G_i is the genotype effect and E_j is the environment effect; λ_k is the singular value for IPCA; γ_{ik} is the genotype G eigenvector value for IPC axis N; δ_{jk} is the environment E eigenvector value for IPC axis N, ρ_{ij} is the interaction residual, and ε_{ijk} is the random error.

The results of the AMMI analysis were interpreted on the basis of AMMI-1 graph, which shows the *nominal yield* (expected yield from the AMMI model equation without environmental deviations) of genotypes across environmental IPCA1 scores (Gauch and Zobel 1997), and AMMI-2 biplot, which shows its IPCA1 on the abscissa and IPCA2 on the ordinate. The AMMI statistic coefficient (D) as suggested by Zhang et al. (1998) was also used to assess the stability of the genotypes.

$$D = \sqrt{\sum_{k=1}^{N} \gamma_i^2} \qquad (i = 1, 2, 3, \ldots, n)$$

where *D* is the distance of interaction principal component (IPC) point with origin in space, N is the number of the significant IPCs and γ is the scores of genotype *i* in IPCs. The greater the *D* value of a genotype, the greater the distance of the genotype from the origin of IPCs. The genotype with the lowest value of the statistic *D* would be more stable (Fan et al. 2001; Zhang et al. 1998).

GGE Biplot Model The GGE biplot model was introduced by Yan et al. (2000) and employed to analyze GE interaction of grain yield in MET data. The GGE biplot model equation is as follow:

$$Y_{ij} - \overline{Y}_{.j} = \lambda_1 \xi_{i1} \eta_{j1} + \lambda_2 \xi_{i2} \eta_{j2} + \varepsilon_{ij}$$

where Y_{ij} is the average yield of genotype *i* in environment *j*; $\overline{Y}_{.j}$ is the average yield over all genotypes in environment *j*; $\lambda_1 \xi_{i1} \eta_{j1} + \lambda_2 \xi_{i2} \eta_{j2}$ are collectively called the first principal component (PC1) and the second principal component (PC2); $\lambda_1 + \lambda_2$ are the singular values for the first and second principal components, PC1 and PC2, respectively; $\xi_{i1} + \xi_{i2}$ are the PC1 and PC2 scores, respectively, for genotype *i*; $\eta_{j1} + \eta_{j2}$ are the PC1 and PC2 scores, respectively, for environment *j*; and ε_{ij} is the residual of the model associated with the genotype *i* in environment *j*. Thus a GGE biplot is constructed by plotting the PC1 scores against the PC2 scores for each genotype and each environment. The GGE biplot methodology, which is composed of two concepts, the biplot concept (Gabriel 1971) and the GGE concept (Yan et al. 2000), was applied to visually analyze the rainfed durum MET data. This methodology uses a biplot to show the effects of G and GE that are important in genotype evaluation and that are also the sources of variation in GE interaction analysis of MET data (Yan et al. 2000). Using GGE biplot methodology, genotypes can be evaluated for their performance, stability, and adaptation in individual environments and across environments. Simultaneously, environment relationships can be

evaluated and mega-environment can be set up by using the biplots (Yan and Kang 2003). Mega-environment analysis and test location evaluation are two important issues for effective crop variety evaluation through multilocation variety trials. These must be done based on multiyear multilocation variety-trial data, which are usually highly unbalanced (Yan 2015). Accordingly, the durum wheat grain yield data were subject to GE interaction analysis using the GGEbiplot software (Yan 2001) in order to (i) generate graphs showing *which-won-where* patterns for mega-environment analysis, (ii) ranking of genotypes based on yield and stability (iii) comparison of genotypes to an ideal genotype and (iv) evaluate test environments for *discriminating ability* vs. *representativeness view*. Angles between environment vectors were used to judge correlations (similarities/dissimilarities) between pairs of environments. The angles of the environmental vectors to each other in the biplot represent the phenotypic correlation between the environments. The cosine of angle between a pair of environment vectors approximates correlation between them. An acute angle (<90°) indicates a positive correlation; an angle close to 90° indicates the environments are not correlated, whereas an obtuse angle represents a negative relationship.

Genotype by Trait (GT) Biplot Analysis The GT biplot method was used to display the genotype by trait two-way data in a biplot across the environments. The GT biplot, as described by Yan and Rajcan (2002), was constructed by plotting the first principal component (PC1) scores of the genotypes and the traits against their respective scores for the second principal component (PC2) that resulted from singular value decomposition (SVD) of traits-centered to study trait relationships and to identify and characterize superior genotypes. In the GT biplot, a vector is drawn from the biplot origin to each marker of the traits to facilitate visualization of the relationships among the traits. The correlation coefficient between any two traits is approximated by the cosine of the angle between their vectors. Acute angles show a positive correlation, obtuse angles show a negative correlation and right angles no correlation (Yan and Rajcan 2002). The length of the vector describes the discriminating ability of the trait. A short vector may indicate that the trait is not related to other traits, that there is a lack of variation or that it is not suitable for genotype discrimination.

14.2.2.4 Correlation Analysis Among Statistical Models

Correlation Analysis Based on Stability Ranks To investigate relationships between multivariate and univariate (parametric and non-parametric) models in genotype rankings for stability, the Spearman (1904) rank correlation coefficients were calculated between stability ranks obtained from multivariate models i.e. GGE biplot and AMMI models with the stability ranks given to each of the univariate stability measures. The GGE biplot stability rankings were determined as visual ratings on the projections of genotypes on the average environment coordinate (AEC): a smaller projection equated to a better stability ranking (e.g. rank of 1). For AMMI, the stability ranks were obtained by assigning best rank to the genotype

with the lowest AMMI distance (*D*) as described by Zhang et al. (1998). Similarly, ranks were assigned for the univariate parametric and non-parametric stability statistics with the lowest estimated value receiving the rank of 1. For TOP and GAI statistics, ranks were obtained by assigning best rank to the genotype with the highest value. Ranks were assigned for mean yield, with the genotype with the highest yield given a rank of 1.

Correlation Analysis Based on Yield-Stability Ranks Among univariate models which integrate yield with stability performance, the parametric methods such as joint regression analysis (JRA), and the Tai (1971) stability analysis and non-parametric statistics of Kang (1988, 1993) i.e. rank-sum (RS) and yield-stability (YSi) statistic were used for simultaneous selection for yield and stability performance. These procedures were compared with the multivariate models i.e. AMMI and GGE biplot for ranking of genotypes for both yield and stability performances. For these cases, the yield-stability (yield + stability) ranks for each model were assigned as follows:

(a) In JRA, the yield ranks were determined by assigning the best rank to the genotype having the highest regression coefficient and the lowest rank to the genotype having the lowest regression coefficient. Stability rankings were obtained by assigning best rank to the genotype with lowest S^2di; and finally yield-stability ranks were determined as the sum of yield and stability ranks (Alwala et al. 2010).
(b) For the Tai method, the ranks were developed as follows: the yield ranks were assigned by giving the best rank to the genotype having the highest response to environmental effects (α) and the last rank was given to the genotype having lowest α-value. Stability rankings were obtained by assigning best rank to the genotype with the lowest deviation from the linear response (λ); and the yield-stability ranks were determined as the sum of yield and stability ranks.
(c) For the rank-sum (RS) method, the yield ranks were determined by giving the best rank to the genotype having the highest mean yield. Stability ranks were obtained by assigning best rank to the genotype with the lowest Shukla stability variance. Finally, yield-stability ranks were obtained as the sum of yield and stability ranks (Kang 1988).
(d) For YSi statistics, the yield ranks were obtained from the phenotypic adjusted yield data (Kang 1993). Stability rankings were obtained by assigning best rank to the genotype with lowest Shukla stability variance (σ^2); and the yield-stability ranks were determined as the sum of yield ranks and stability ranks (Kang 1993).
(e) For AMMI, the ranks were assigned as follows: (i) the yield ranks were determined by giving the best rank to the genotype having the highest *nominal yield* (expected yield from the AMMI model equation without environmental deviations); (ii) the stability rankings were obtained by assigning best rank to the genotype with the lowest AMMI distance (*D*) and (iii) yield-stability ranks were determined as the sum of yield ranks and stability ranks.

(f) For GGE biplot, the ranks were assigned as follows: (i) the yield ranks were determined by giving the best rank to the ideal genotype which is on the far right hand side and the least was given to the genotype on the far left hand side of the biplot; (ii) stability rankings were determined as visual ratings on the projections of genotypes on the AEC ordinate: a smaller projection equated to a better stability ranking; and (iii) yield-stability ranks were determined as the sum of yield ranks and stability ranks (Alwala et al. 2010).

14.3 Analysis of Data and Panel Discussion

14.3.1 Partitioning GE Components of Variability

The combined analysis of variance on grain yield data revealed that main effects due to environment, genotype and GE interaction were found to be highly significant ($P<0.01$) (Table 14.1). The environments accounted for 94 % of total variation, followed by GE interaction which captured 5.2 % and genotype effect accounted for 0.7 %. The significance of the GE interaction effect suggests that there are significant differences in responses of genotypes to environments, and hence sensitivity and instability. The greater GE interaction relative to genotype effect suggests significant environmental groups with different top-yielding genotypes. The large variation due to environment confirms that the testing environments were different, with large differences among environmental means causing most of the variation in genotypic performances (Fan et al. 2007; Yan and Kang 2003). Genotypic rank differences over environments showed the existence of crossover GE interaction (Crossa 1990), which showed the necessity to assess the response of the genotypes to environmental variation. The linear regression accounted for 27.7 % of GE interaction variation, whereas the residual of the variation around regression slope explained 72.3 % of variation (Table 14.1). A large portion of GE interaction was due to a non-linear component which can be regarded as a very important parameter for selection of stable genotypes. Analysis of multiplicative effects indicated that the first four IPCAs were found to be highly significant ($P<0.01$). A breakdown of the GE interaction into the first four IPCAs (IPCA1 to IPCA4) shows that the GE interaction sum of square (SS) was spread in decreasing order of magnitude of 50.3, 15.1, 10.8 and 8.6 %, respectively, of the total GE interaction SS.

14.3.2 Genotype Evaluation and Selection

14.3.2.1 Univariate Stability Statistics

The estimates of phenotypic-stability statistics for the 14 tested genotypes and the genotypic ranks based on the stability statistics are given in Table 14.2. Taking the mean yield as the first priority for evaluating the entries, breeding line G8 followed

Table 14.1 Combined analysis of variance, AMMI and joint regression analyses of grain yield of 14 genotypes grown across 16 diversified environments

Sources of variation	Df	MS	% TSS	% GE interaction
Genotype	13	204,018**	0.7	
Environment	15	22,508,400**	94	
GE interaction	195	96,649**	5.2	
Regression	13	400,963**		27.7
Deviation	182	74,913**		72.3
IPC 1	27	351,309**		50.3
IPC 2	25	113,573**		15.1
IPC 3	23	88,359**		10.8
IPC 4	21	77,413**		8.6
GE residual	99			15.2
Total	223			

MS mean squares, *%TSS* percentage relative to total sum of squares
**Significant at 1 % level of probability

by G12 (new cultivar), G11 and G4 gave the best mean yield, while G13 (durum old landrace) followed by G14 (bread wheat, old landrace), G3 and G1 had the lowest mean yield across environments.

Regression analysis on grain yield of 14 entries evaluated in 16 diverse environments were performed to assess the nature of GE interaction and to assess their relative stability performance. The lines G1, G12, G10, G6, and G11 with regression coefficient approximating 1.0 tended to have general stability (Table 14.2). These lines had low variance in regression deviation (S^2di). The breeding lines G4, G7 and G8 with $b > 1$ were more adapted to favorable environments. These genotypes with high variance in regression deviation had stability below average. The two old varieties (G13 and G14) with the lowest b values were more adapted to unfavorable environments and with the highest variance in regression deviations showing highest instability.

The Tai (1971) model is based on the principle of structural relationship analyses, in which the GE interaction effect of genotype is partitioned into two components. According to the alpha (α) and lambda (λ) stability statistics, genotypes G12, G11, G1, G6, G10 and G3 could be considered as having average stability (Table 14.2) because these genotypes had values close to (α, λ) = (0,1), while G14 and G13 were significantly unstable. In the durum MET data the entries differed statistically in the amount of deviation from the linear response (λ) and the magnitude of linear response (α). The distributions of α and λ values of the 14 entries are shown in Fig. 14.1. The average stability region in the Fig. 14.1 contains the G12, G11, G1, G6, G10, G3 and G7. Among these genotypes G12 and G11 were superior yielders. The highest-yielding genotype among tested entries, G8, was relatively unstable. In contrast to G8, both old varieties (G14 and G13) with lowest yield productivity gave a very unstable performance.

Table 14.2 Mean yield, phenotypic stability measures and stability ranks for 14 genotypes across 16 environments

Code	Mean yield	Parametric stability measures									Non-parametric stability measures						
		Joint regression		Tai (1971) estimates													
		bi	S^2di ($\times10^{-3}$)	α	λ	σ^2 ($\times10^{-3}$)	S^2x ($\times10^{-3}$)	*D*	GAI	Pi ($\times10^{-4}$)	$S_i^{(1)}$	$S_i^{(2)}$	$S_i^{(3)}$	$S_i^{(6)}$	TOP	RS	YSi
G1	2161	1.00	55.5	–0.001	1.426	51.8	1656	20.3	1726	21.7	1.85	3.77	25.5	5.97	31	19	2
G2	2212	1.08	43.3	0.078	1.112	50.0	1907	17.1	1765	16.0	1.72	3.5	20.7	4.99	19	17	3
G3	2155	1.06	38.9	0.061	0.998	42.2	1845	8.5	1685	21.1	1.74	3.45	20.2	4.92	13	16	3
G4	2292	1.11	83.0	0.110	2.132	96.8	2057	27.8	1758	12.7	1.84	4.45	36.7	7.45	31	15	3
G5	2271	1.06	79.2	0.061	2.035	79.9	1884	23.9	1832	15.3	1.88	4.18	38.5	8.02	44	14.5	2
G6	2230	0.97	34.6	–0.030	0.891	33.8	1546	15.1	1828	14.6	1.74	3.47	22.8	5.69	31	12	6
G7	2237	1.10	70.2	0.095	1.807	80.0	1993	22.9	1755	15.9	1.79	3.91	29.5	6.97	38	18	–1
G8	2418	1.10	114.0	0.101	2.929	122.5	2052	30.1	1969	10.5	1.89	4.09	49.6	10.89	56	13	6
G9	2258	1.07	22.0	0.066	0.563	27.6	1848	14.6	1795	14.0	1.64	3.4	21.8	5.67	19	9	8
G10	2271	0.98	23.6	–0.020	0.605	22.6	1566	11.7	1893	13.7	1.51	2.96	19.9	6.04	38	6.5	9
G11	2343	1.03	46.2	0.029	1.186	44.4	1744	11.8	1917	10.3	1.82	3.77	34.1	8	50	8	12
G12	2397	1.01	52.5	0.011	1.347	49.2	1692	16.3	2041	7.8	1.34	2.83	24.7	7.47	69	8	11
G13	2018	0.79	137.8	–0.212	3.540	200.5	1129	33.2	1725	40.8	1.91	4.65	32	6.25	25	27	–7
G14	2065	0.65	173.2	–0.348	4.450	355.2	847	42.7	1876	44.6	1.95	5.39	58.1	10.4	38	27	–6
Rank																	
G1	11	1	8	1	7	8	5	1	12	12	10	7.5	7	5	9	12	10.5
G2	10	9	5	9	3	7	11	10	9	10	4	6	3	2	12.5	10	8
G3	12	6.5	4	6.5	1	4	8	2	14	11	5.5	4	2	1	14	9	8
G4	4	12	11	12	11	11	14	11	10	4	9	12	11	9	9	8	8
G5	5.5	6.5	10	6.5	10	9	10	7	6	8	11	11	12	12	4	7	10.5
G6	9	4.5	3	5	2	3	3	5	7	7	5.5	5	5	4	9	5	5.5

(continued)

Table 14.2 (continued)

Code	Mean yield	Parametric stability measures									Non-parametric stability measures						
		Joint regression		Tai (1971) estimates													
		*b*i	S^2di ($\times10^{-3}$)	α	λ	σ^2 ($\times10^{-3}$)	S^2x ($\times10^{-3}$)	*D*	GAI	Pi ($\times10^{-4}$)	$S_i^{(1)}$	$S_i^{(2)}$	$S_i^{(3)}$	$S_i^{(6)}$	TOP	RS	YSi
G7	8	10.5	9	10	9	10	12	9	11	9	7	9	8	8	6	11	12
G8	1	10.5	12	11	12	12	13	12	2	3	12	10	13	14	2	6	5.5
G9	7	8	1	8	8	2	9	4	8	6	3	3	4	3	12.5	4	4
G10	5.5	3	2	3	6	1	4	3	4	5	2	2	1	6	6	1	3
G11	3	4.5	6	4	4	5	7	8	3	2	8	7.5	10	11	3	2.5	1
G12	2	2	7	2	5	6	6	6	1	1	1	1	6	10	1	2.5	2
G13	14	13	13	13	13	13	2	13	13	13	13	13	9	7	11	13.5	14
G14	13	14	14	14	14	14	1	14	5	14	14	14	14	13	6	13.5	13

YLD mean yield, *b* regression coefficient, S^2di variance in regression deviation, *α* alpha, *λ* lambda, σ^2 Shukla (1972) stability variance, S^2x environmental variance, *D* AMMI statistic coefficient, *GAI* geometric stability index, *Pi* superiority index, $S_1^{(i)}$, $S_2^{(i)}$, $S_3^{(i)}$, $S_6^{(i)}$ Huehn (1990) non-parametric stability statistics, *TOP* Fox et al. (1990) non-parametric statistic, *RS* Kang (1988) rank sum, *YSi* yield-stability index

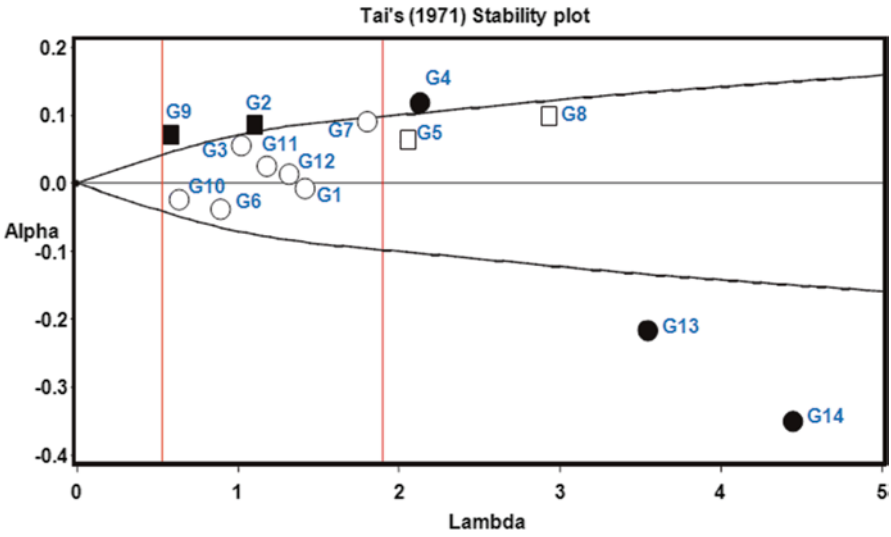

Fig. 14.1 Distributions of Tai (1971) stability statistics of the 14 genotypes grown in 16 environments in durum MET data. *Solid circles*: both Alpha and Lambda are significant; *open circles* both Alpha (α) and Lambda (λ) are not significant; *solid squares*: only Alpha is significant; *open squares*: only Lambda is significant. G1–G14 are genotypic codes; G1–G11 breeding lines; G12: new cultivar; G13: old durum variety; G14: bread wheat old variety

The Shukla (1972) stability variance indicated that the genotypes G10, G9, G6 and G3 had lower variance and could be considered stable, whereas the two old varieties (G14 and G13) followed by G8 and G4 were more unstable. According to the Lin and Binns (1988) superiority index (Pi), the genotypes G12, G11 and G8 with low Pi values indicated high relative stability and these genotypes also had high grain yield productivity. In relation to this method, the landraces G14 and G13 and line G1 with high Pi value indicate low relative stability. These results were similar to those obtained by genotype rankings using the Shukla (1972) stability variance parameter. Based on the environmental variance, S^2x, the entries G14, G13 and G6 with minimum variance across environments were considered to be stable and the genotypes G4, G8 and G7 unstable (Table 14.2). The estimates of AMMI statistic coefficient (*D*) varied between genotypes, ranging from 8.5 to 42.7 (Table 14.2). Genotype G3 with the lowest *D* value was the most stable genotype followed by G10, G11, G9 and G6, while the old varieties (G14 and G13) with highest value of *D* statistic were the most unstable genotypes. According to GAI (Mohammadi and Amri 2008) genotypes G12, G8 and G11 with high values were adapted to most of the environments and the entries G3, G13 and G1 were poorly adapted to most of the environments (Table 14.2).

Two rank stability methods ($S_i^{(1)}$ and $S_i^{(2)}$) from Nassar and Huehn (1987) are based on ranks of genotypes across environments and they give equal weight to each environment. Genotypes with fewer changes in ranking are considered to be more

stable (Becker and Leon 1988). Genotypes with less change in ranks are expected to be more stable. Accordingly, $S_i^{(1)}$ and $S_i^{(2)}$ of the tested genotypes showed that genotypes G12, G10, G9 and G2 had the lowest values; therefore, these genotypes were regarded as the most stable genotypes according to $Si^{(1)}$ and $Si^{(2)}$. On the other hand, G14, G13, G8, and G5 had the highest $Si^{(1)}$ and $Si^{(2)}$ values, and therefore, they were determined to be the most unstable.

Two other non-parametric statistics of Huehn (1979), $S_i^{(3)}$ and $S_i^{(6)}$ combine yield and stability based on yield ranks of genotypes in each environment. These parameters measure stability in units of the mean rank of each genotype (Huehn 1979). The lowest value for each of these statistics indicates maximum stability for a specific genotype. Based on the statistic $Si^{(3)}$, breeding lines G10, G3, G2 and G9 can be considered as high stable genotypes, while G14, G8, G5 and G4 can be regarded as unstable genotypes. As for $Si^{(3)}$, the G3, G2, G9 and G6 were found as stable genotypes based on the $Si^{(6)}$ and the G8, G14, G5 and G11 as unstable genotypes. The G8 with the highest mean yield among the genotypes tested was an unstable genotype, while G12 as second in mean yield was stable (Table 14.2).

According to the TOP parameter of Fox et al. (1990), G12 was an adapted genotype because it ranked in the top third of genotypes in a high percentage of environments (high top value, 69 %), followed by G8 (56 %) and G11 (50 %) (Table 14.2). The undesirable genotypes identified by this method (TOP) were G3, G9 and G2; and the MID values for these genotypes were 44, 44 and 38 %, respectively (data not shown). The genotypes with the highest values of TOP had the lowest LOW values.

The RS (Kang 1988) is a non-parametric stability statistic which uses both yield and Shukla's stability variance (Shukla 1972). The genotypes with the lowest RS are the most favorable genotypes. According to the RS statistic, G10, G11, G12, G9 and G6 had the lowest values and therefore were stable genotypes with high yield (Table 14.2), while G14, G13, G1, G7 and G2 were undesirable.

The YSi statistic, which integrates yield with stability, varied from −7 to 12 across 16 environments (Table 14.2). Based on the YSi statistic, G11, G12, G10, G9 and G6 were identified as the top 5 desirable genotypes for integrating yield with stability performance, while G13, G14, G7, G5 and G1 were undesirable. In addition, breeding line G8 with YSi values > mean = 3.6 can be selected as a desirable genotype.

Relationships Among Univariate Stability Statistics Each of the abovementioned stability statistics produced a genotype order (Table 14.2). The Spearman (1904) rank correlation analysis was performed between the stability statistics to identify relationships among the methods in genotype rankings (Table 14.3). The most prominent relationships were strong positive correlations between Pi, YSi, TOP, RS and GAI. These parameters were closely correlated with mean yield and related to the dynamic concept of stability. In the dynamic concept of stability, it was not required that the genotypic response to environmental conditions be equal for all genotypes (Becker and Leon 1988). Thus, stable genotypes according to these parameters were recommended for growing in favorable conditions of Iran.

Table 14.3 The Spearman (1904) rank correlation coefficients between mean yield and stability parametric and non-parametric statistics for 14 genotypes across 16 environments

	YLD	b	S^2di	α	λ	σ^2	S^2x	GAI	Pi	YSi	$S_1^{(i)}$	$S_2^{(i)}$	$S_3^{(i)}$	$S_6^{(i)}$	TOP
b	0.28														
S^2di	0.10	0.63*													
α	0.28	0.99**	0.63*												
λ	0.03	0.68**	0.81**	0.68**											
σ^2	0.18	0.72**	0.98**	0.72**	0.80**										
S^2x	−0.53	0.20	0.03	0.19	0.03	0.10									
GAI	0.72**	0.24	0.02	0.23	−0.05	0.12	0.037								
Pi	0.95**	0.36	0.28	0.36	0.21	0.37	−0.402	0.69**							
YSi	0.71**	0.59*	0.65*	0.58*	0.55*	0.72**	−0.115	0.63*	0.85**						
$S_1^{(i)}$	0.28	0.58*	0.85**	0.58*	0.73**	0.84**	−0.088	0.16	0.46	0.68**					
$S_2^{(i)}$	0.26	0.74**	0.88**	0.74**	0.77**	0.89**	0.055	0.21	0.43	0.73**	0.93**				
$S_3^{(i)}$	−0.18	0.52	0.86**	0.52	0.76**	0.82**	0.125	−0.28	0.01	0.37	0.83**	0.84**			
$S_6^{(i)}$	−0.49	0.27	0.71**	0.27	0.65*	0.60*	0.081	−0.63*	−0.31	0.04	0.56*	0.55*	0.87**		
TOP	0.70**	0.20	−0.34	0.20	−0.26	−0.21	−0.047	0.81**	0.58*	0.32	−0.13	−0.08	−0.52	−0.85**	
RS	0.76**	0.58*	0.62*	0.57*	0.44	0.72**	−0.112	0.67**	0.87**	0.94**	0.66*	0.69*	0.33	−0.04	0.40

YLD mean yield, *b* regression coefficient, S^2di variance in regression deviation, *α* alpha, *λ* lambda, σ^2 the Shukla (1972) stability variance, S^2x environmental variance, *GAI* geometric stability index, *Pi* superiority index, $S_1^{(i)}$, $S_2^{(i)}$, $S_3^{(i)}$, $S_6^{(i)}$ the Huehn (1996) non-parametric stability statistics, *TOP* Fox et al. (1990) non-parametric statistic, *RS* the Kang (1988) rank sum, *YSi* yield-stability index

*, **Significant at 5 % and 1 % level of probability

The rest of stability parameters including the Huhen (1979) non-parametric measures, JRA parameters and the Tai stability parameters were correlated with each other, while they were not associated with mean yield, showing they can be regarded as static (biological) parameters for selecting stable genotypes. In the static concept of stability, a genotype which shows a constant performance in all environments does not necessarily respond to improved growing conditions with increased yield. Therefore, stable genotypes according to these methods are recommended for regions where growing conditions are unfavorable. The joint regression parameters were closely and positively associated with the Tai (1971) parameters (α and λ), the Shukla (1972) stability variance (σ^2) and the Huehn (1979) non-parametric measures ($S_1^{(i)}$ and $S_2^{(i)}$) in genotype rankings, indicating that they can be used in stability analysis interchangeably. $S_3^{(i)}$ and $S_6^{(i)}$ were closely associated ($P<0.01$) with S^2di. S^2x was not associated with each of the other stability statistics. The S^2x, $S_3^{(i)}$ and $S_6^{(i)}$ were the only methods which showed negative correlations with mean yield but not significant, indicating these methods are suitable to identify low yielding stable genotypes, thus they may be ignored for stability analysis.

Rank correlation among stability parameters indicated that they can be classified into groups related to static and dynamic concepts of stability (Becker and Leon 1988). Based on this, significant genetic improvement was observed for the promising breeding lines compared to the controls for both concepts of stability. The stability parameters related to static stability would be useful if selection was to be based primarily on stability. Stable genotypes (i.e. G10, G3, G9) based on this group would be suited to unfavorable environments that have poor edaphic and climatic conditions. In contrast, those related to a dynamic concept of stability would be more useful for breeders interested primarily in yield. Genotypes with dynamic stability (i.e. G12, G11, G8) would be recommended for unpredictable and/or favorable environments. Therefore, breeders can recommend the best adapted genotypes using the methods related to the dynamic concept of stability (Flores et al. 1998; Fox et al. 1990; Mohammadi and Amri 2008). In contrast, static stability may be more useful than dynamic in a wide range of situations, especially in developing countries (Simmonds 1991). The stability parameters verified that the genotypes could be grouped based on yield and stability performance. Such groupings are useful to breeders in identifying promising candidate lines that may be released as new cultivars or be used as parents in the breeding programs. The old varieties were outyielded by modern cultivar as well as breeding lines in both yield and yield stability, which agree with other studies (Donmez et al. 2001; Morgounov et al. 2010; Ortiz et al. 2001; Pswarayi et al. 2008; Tollenaar and Lee 2002; Xiao et al. 2012).

14.3.2.2 Multivariate Statistical Models

AMMI Biplot Analysis of MET Data Genotype nominal yields, estimated on the basis of the AMMI model equation without the environmental deviation across environment IPCA1 scores, indicate the adaptability of each genotype (Gauch and Zobel 1997). This information allows for the evaluation of the effects of genetic

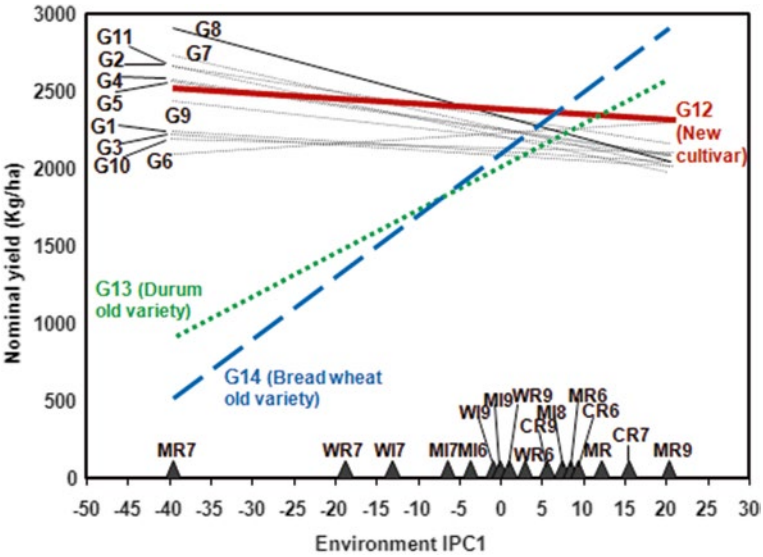

Fig. 14.2 Nominal yield (kg ha^{-1}) of 14 genotypes as a function of the score on the environment IPCA1 scores of 16 divergent environments. The lines are the responses of genotypes to different environments and the *black triangles* are the environments which are ranked base on their IPCA1. G1–G14 are genotypic codes. G1–G11 breeding lines; G12: new cultivar; G13: old durum variety; G14: bread wheat old variety

improvement on yield stability and adaptability and the identification of the highest yielding genotypes in specific environment IPCA1 ranges. The lines in Fig. 14.2 result from the projection of the nominal yield of each entry versus the IPCA1 scores of the environments. The order of the environments along the IPCA1 axis suggested that the climatic conditions (rainfall and temperatures), had a greater impact on the occurrence of GE interaction. The slope of the lines reflect the adaptation patterns of the genotypes across the environmental IPCA1 scores. The results show that these interactions lead to different rankings of the genotypes across the environments. From Fig. 14.2, the two old varieties (G13 and G14) showed a sharp slope (highest instability) and exhibited the lowest nominal yield in environments with large negative IPCA1 and the highest nominal yield at environments with the large positive IPCA1. In contrast, the breeding lines G8, G7 and G11 had low slope and exhibited the highest nominal yield in the environments with large negative IPCA1 and average yield at environments with large positive IPCA1. This indicates that the breeding lines G8, G7 and G11 are in contrast with the two old varieties in adaptation, yield performance and stability. The G12 (cv. Saji) had high nominal yield levels across the whole range of environment IPCA1 scores representing the best adapted genotype within the entries tested. The genotypes G1, G3 and G10 had average nominal yield over the environmental IPCA1 scores with the highest stability performance. With respect to the most discriminating environments (MR7 and MR9), the nominal yield ranged from 2333 to 2516 kg ha^{-1} for G12 as highly

adapted genotype; 937–2593 and 521–2885 kg ha^{-1} for the two landraces (G13 and G14), respectively, as genotypes with the highest instability. As shown in Fig. 14.2, the warm and some moderate cold environments with negative IPCA1 are clearly separated from cold and other moderate cold environments. It can be concluded that the two old varieties are highly adapted to cold environments while the breeding lines G8, G7, G2 and G11 are highly adapted to warm and moderate cold environments, and the rest of entries with general adaptability to all environments had average combination of yield and stability.

In breeding for wide adaptation, the aim is to obtain a variety which performs well in nearly all environments, while in breeding for specific adaptation, the aim is to obtain a variety which performs well in a definite subset of environments within a target region (Annicchiarico 2002). However, GE interaction can be exploited by specific adaptation, or minimized by wide adaptation. These two strategies are shown graphically in Fig. 14.3 with respect to three high yielding genotypes that differ for adaptive response in durum MET data. Minimizing GE interaction effects by growing the cultivar with lowest GE interaction over all the region, i.e. modern cultivar, implies a yield penalty relative to growing specifically-adapted germplasm, i.e. Sardari bread wheat (G14) in the low-yielding sub-region (cold environments) along with breeding line G8 in the high-yielding sub-region (warm environments). Likewise, aiming at selecting a variety like modern cultivar in the context of a wide-adaptation strategy may imply lower yield gains over the region than breeding distinct germplasm for each sub-region. However, the choice between exploiting and minimizing the GE interaction effects requires assessment of the yield gains at parity of costs.

To graphically analyze GE interaction and sensitivity degree between the genotypes and environments, the AMMI-2 biplot based on the IPCA1 and IPCA2 scores are displayed in Fig. 14.4. In the AMMI-2 biplot (Fig. 14.4) points situated close to the origin with scores close to zero for the IPCA1 and IPCA2 axes represent stable genotypes and environments. The interpretation of AMMI-2 biplot for GE interaction is based on the magnitude and on the signs of the scores of the genotypes or environments for the interaction axes considered. The relative position of the genotypes versus the environmental vectors is based on their interaction. The GE biplot illustrates the role of the IPCA on the performance of genotypes and environments through the distance of each genotype or environment from the origin. In the biplot, the long vectors corresponding to the moderate (i.e. MR7, MI7, MI9, MR9 and MI6) and warm (WR7) environments indicated that these environments were likely to have a great influence in determination of GE interaction. The short vectors corresponding to warm and cold environments showing they tend to contribute less to GE interaction, resulting in poor genotype discrimination.

The angles between the environments CR6, CR7 and CR9 (corresponding to cold location) well below 90°, indicate that the cold environments tend to discriminate the genotypes in the same direction. These environments tended to discriminate the two old varieties Sardari (G14) and Zardak (G13). A wide variation was found between the moderate environments in association with the cold and warm environments including for genotype discrimination. Four out of eight moderate environ-

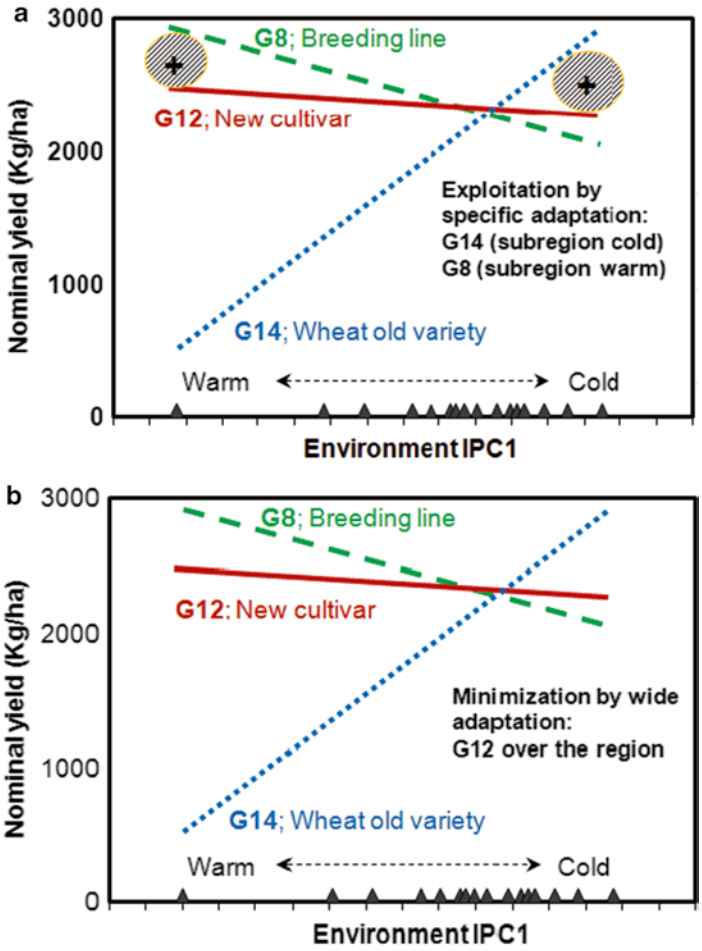

Fig. 14.3 Exploitation by specific adaptation (**a**), and minimization by wide adaptation (**b**) of GE interaction for three high yielding genotypes that differ for adaptive response in durum MET data

ments (i.e. MR8, MR6, MR9 and MI8) were highly associated with the cold environments. The two moderate irrigated environments of MI6 and MI9 tend to highly discriminate the genotypes G4, G12 and G6. Genotype G8 was also highly adapted to moderate irrigated environment MI7. The two breeding lines G2 and G7 performed well at the warm (WI7) and moderate (MI7) irrigated environments. The genotype G10 had the best performance at the warm environments (WR9, WR6 and WI9). The analysis of the genotypic responses in AMMI-2 biplot (Fig. 14.4) indicates that the two old varieties (G13 and G14) were grouped apart from breeding lines that were mostly adapted to warm and moderate environments. The breeding lines can be classified into two different groups. The first group included the genotypes G1, G3, G9, G5 and G10 with the smallest IPCA1 and IPCA2 scores and the lowest contribution to GE interaction, thus they tend to have general stability. The second group with the highest IPCA1 or/and IPCA2 had the highest contribution to

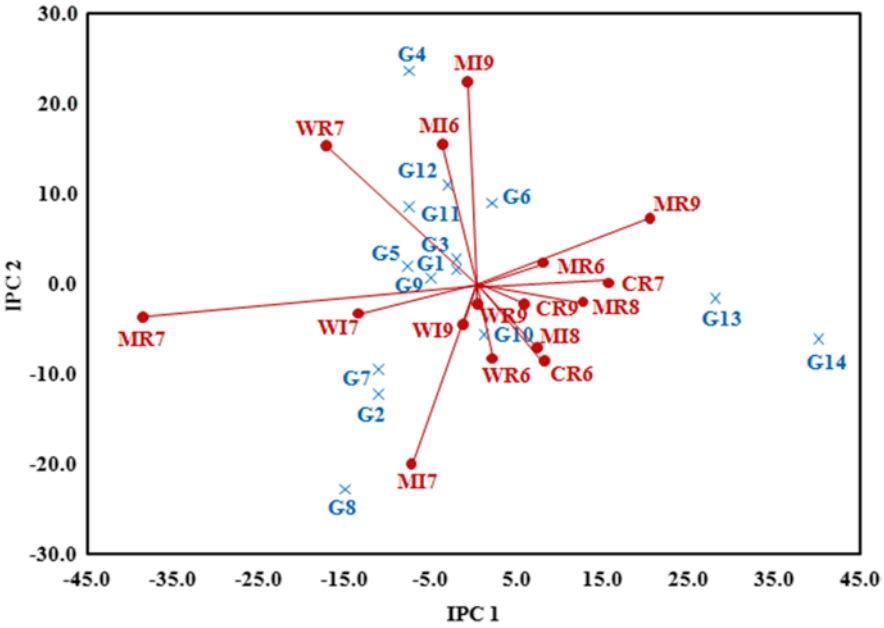

Fig. 14.4 AMMI-2 biplot derived from the first two IPCAs for 14 genotypes across 16 environments. The vectors are the testing environments. The letters M, W and C included in the initial of environmental names are stand for moderate, warm and cold locations, respectively; the letters R and I included in the environmental names are stand for rainfed and irrigated conditions, respectively; and the number of 6, 7, 8 and 9 included in the environmental names stand for 2005–2006, 2006–2007, 2007–2008 and 2008–2009 cropping seasons, respectively. G1–G14 are genotypic codes; G1–G11 breeding lines; G12: new cultivar; G13: old durum variety; G14: bread wheat old variety

GE interaction, thus they tend to have specific adaptation. Among these genotypes, the G4 and G8 had specific adaptation to some of the moderate and warm environments, while the two old varieties were more adapted to cold environments and some of the other moderate environments.

Based on the results, significant yield improvements in warm and moderate locations, and yield stability improvement across environments in comparison to the controls, were obtained. Genetic gains in yield were observed mostly in non-stressed environments in each location. This agrees with other published results (Ceccarelli 1996; Donmez et al. 2001; Mohammadi and Amri 2009; Munoz et al. 1998; Pswarayi et al. 2008). Although, these differences could be attributed to differences in the genetic material tested as well as to differences in the testing environments, the studied genotypes showed considerable variability in stability and adaptation to different climatic conditions, that could be exploited in crop improvement. However, environments that are similar in terms of genotype response can be grouped by different methods (Annicchiarico 2002), and each group may identify a cropping area that is relatively uniform because genotype x location interaction effects are limited or negligible. Such areas have been termed by different authors as subregions (Horner and Frey 1957; Seif et al. 1979), subzones (Annicchiarico 2002), subareas

(Annicchiarico 2002), macro-environments (Ceccarelli 1989) or mega-environments (Yan et al. 2000). The results from this study suggest both that there are wide and specific adaptations in the tested entries. Based on this, genotypes with specific adaptation can be recommended for each of the warm and cold subregions and genotypes with wide adaptation can be recommended for areas with different environmental conditions. Different subregions may be identified not only within large or transnational regions (Crossa et al. 1991; DeLacy et al. 1994; Mohammadi et al. 2010a, b, 2012) but also within relatively small regions, as suggested by the results from northern Syria (Ceccarelli 1996); Italy (Annicchiarico 1997) and northern Italy (Annicchiarico 2002); New South Wales (Basford and Cooper 1998; Seif et al. 1979); Queensland (DeLacy et al. 1996); southwest Canada (Saindon and Schaalje 1993); Ontario (Yan et al. 2000); and western Iran (Mohammadi and Haghparast 2011). Therefore, the choice between a wide and a specific adaptation strategy may be a key question for national breeding programs (Annicchiarico 2002).

GGE Biplot Analysis of MET Data The polygon view of GGE biplot (Fig. 14.5) provides the best way to visualize the interaction patterns between genotypes and

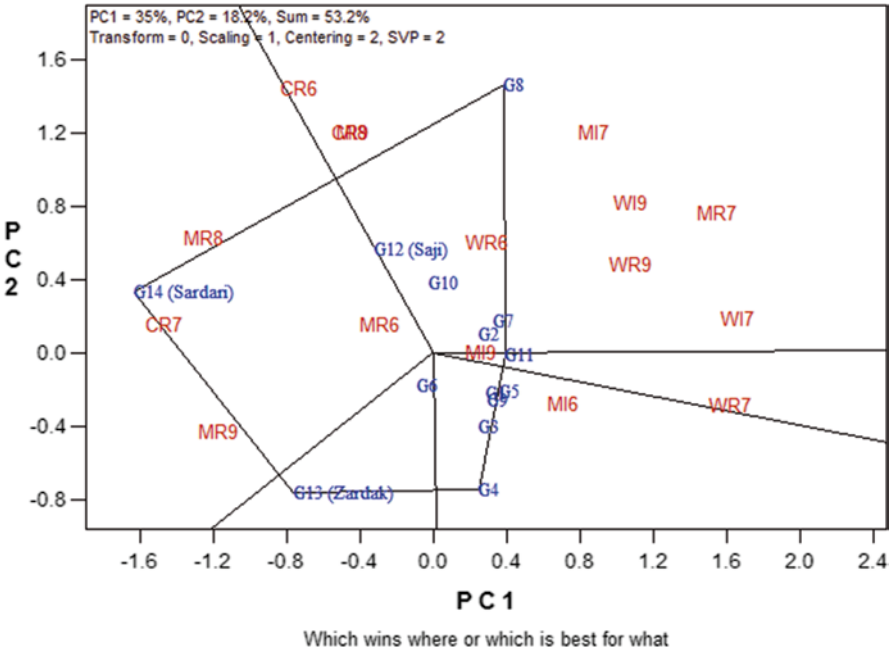

Fig. 14.5 Polygon view of GGE biplot for 14 genotypes across 16 diversified environments. The letters M, W and C included in the initial of environmental names stand for moderate, warm and cold locations, respectively; the letters R and I included in the environmental names are stand for rainfed and irrigated conditions, respectively; and the number of 6, 7, 8 and 9 included in the environmental names stand for 2005–2006, 2006–2007, 2007–2008 and 2008–2009 cropping seasons, respectively. G1–G14 are genotypic codes; G1–G11 breeding lines; G12: new cultivar; G13: old durum variety; G14: bread wheat old variety

environments and to effectively interpret a biplot. The which-won-where graph is constructed first by joining the farthest genotypes forming a polygon. Subsequently perpendicular lines are drawn from the origin of the biplot to each side of the polygon, separating the biplot into several sectors with one genotype at the vertex of the polygon. Genotypes at the vertices of the polygon are either the best or poorest in one or more environments. The genotype at the vertex of the polygon performs best in the environment falling within the sectors (Yan 2002; Yan and Tinker 2005). The biplot indicated the existence of crossover GE interaction and the existence of mega-environments in MET data. Environments that fall into different sectors have different best genotypes. Genotypes located near the biplot origin are less responsive to the change of environments. The vertex entries in this investigation were G8, G14 (bread wheat, old variety), G13 (durum wheat, old variety) and breeding lines G4 and G11. These genotypes were the best or the poorest genotypes in some or all of the environments since they had the longest distance from the origin of the biplot (Yan and Kang 2003).

The environments fell into four and the genotypes into five sections. G14 was more adapted to the cold (CR7, CR6) and moderate cold (MR8, MR9 and MR6) environments. The breeding line G8 performed well at warm (WR6, WR9, WI7), moderate (MI7, MR7, MI9) and cold (CR9) environments, while the breeding line G11 was adapted to warm (WR7) and moderate (MI9) environments and the breeding line G4 performed well at moderate (MI6) environment. G13 was not the best yielding genotype at any of the environments. The G8 and G14 with the greatest distance from the origin of biplot had the highest contribution to GE interaction. These two genotypes were adapted to 13 out of 16 test environments. These two genotypes were diverse in their adaptations, because G14 is the bread wheat old variety which is highly adapted to cold and moderate cold rainfed areas of Iran and it is not recommend of use in warm areas (Mohammadi et al. 2010a, b), while G8 shows high adaptation to environments representing warm areas. In contrast, G6 with the least distance from the origin of biplot tend to contribute least to GE interaction and can be considered as genotype with general adaptation. The environments corresponding to moderate cold location tend to group with the environments corresponding to both cold and warm locations. This is well documented that the moderate cold location from year to year could change from cold to warm (Mohammadi et al. 2010a).

Figure 14.6 shows the ranking of the 14 entries based on their mean yield and stability performance across 16 environments. The line passing through the biplot origin is called the average environment coordinate (AEC), which is defined by the average PC1 and PC2 scores of all environments (Yan and Kang 2003). The closer to a concentric circle the higher the mean yield. The line which passes through the origin and is perpendicular to the AEC with double arrows represents the stability of genotypes. Either direction away from the biplot origin, on this axis, indicates greater GE interaction and reduced stability. According to Fig. 14.6, the breeding line G8 followed by G12 and G10 were identified as genotypes with high yield and stability performances. The other genotypes on the above side of the line with dou-

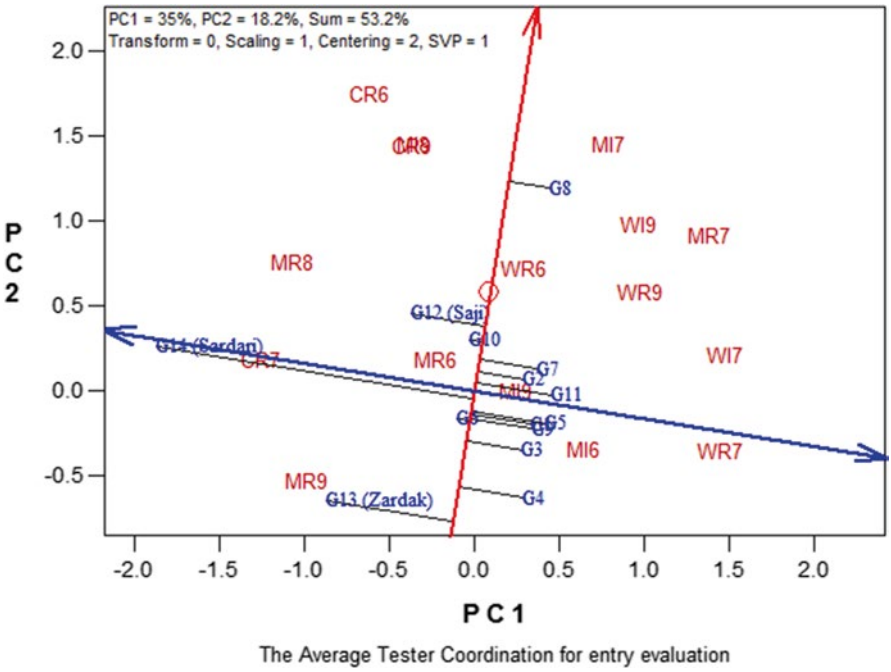

Fig. 14.6 GGE biplot showing the ranks of genotypes based on both yield and stability performance. The letters M, W and C included in the initial of environmental names stand for moderate, warm and cold locations, respectively; the letters R and I included in the environmental names stand for rainfed and irrigated conditions, respectively; and the number of 6, 7, 8 and 9 included in the environmental names stand for 2005–2006, 2006–2007, 2007–2008 and 2008–2009 cropping seasons, respectively. G1–G14 are genotypic codes; G1–G11 breeding lines; G12: new cultivar; G13: old durum variety; G14: bread wheat old variety

ble arrows have yield performance greater than mean yield and those genotypes on the low side of this line had lesser yield than mean. The durum (G13) and bread (G14) wheat old varieties had low yield and stability performances. These results showed that most of the breeding lines were more stable and higher yielding than the old varieties. These results also confirmed the superiority of G8 over G12 (a newly released durum variety for moderate rainfed regions of Iran) and it can be a good candidate for commercial release in rainfed areas of Iran, where the durum wheat is grown.

An ideal genotype should have the highest mean performance and be stable. Although such an ideal genotype may not exist in reality, it can be used as a reference for genotype evaluation (Yan and Kang 2003). Thus, using the ideal genotype as the center, concentric circles were drawn to help visualize the distance between each genotype and the ideal genotype (Fig. 14.7). An ideal genotype, which is located at the center of the concentric circles, is the one that has both high mean yield and high stability. Therefore, G8 can be regarded as an ideal genotype. The genotypes G12 and G10 were near to the ideal genotype. Ranking of other

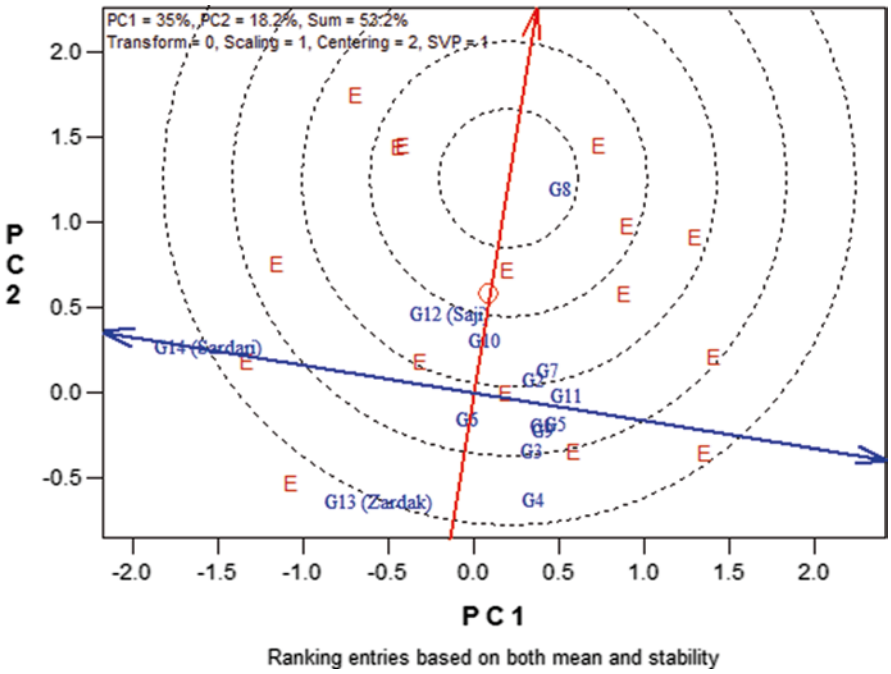

Fig. 14.7 GGE biplot which shows the evaluation of genotypes based on an ideal genotype. G1–G14 are genotypic codes. G1–G11 breeding lines; G12: new cultivar; G13: old durum variety; G14: bread wheat old variety

genotypes based on the ideal genotype was G7 > G2 > G11. The entries G13, G4 and G3 were unfavorable because they were far away from the ideal genotype.

The test environment evaluation has become an increasingly important issue in plant breeding (Yan and Holland 2010). Although MET data are used for genotype evaluation, they can also be used in environment evaluation. An ideal environment should be highly differentiating among genotypes and at the same time be representative of the target area. Discriminating ability refers to an environmental ability to maximize the variance among genotypes in a study (Blanche and Myers 2006). Representativeness suggests that an environment is representative of the conditions of other environments included in the study. An ideal test of environment combines both of these aspects for the development of generally-adapted germplasm. In Fig. 14.8, the environments are ranked based on both discriminating ability and representativeness. The small circle is where an ideal environment should be; its projection on the AEC x-axis was designed to be equal to the longest vector of all environments; therefore, it is the most discriminating; its projection on the AEC y-axis was obviously zero, meaning that it is absolutely representative of the average environment. Accordingly, the environment WR6 was the most representative environment followed by MI7, MI8 and CR9, while the environment MR7 was the most discriminative one followed by CR6, WI7 and WR7. In the case of both discrimina-

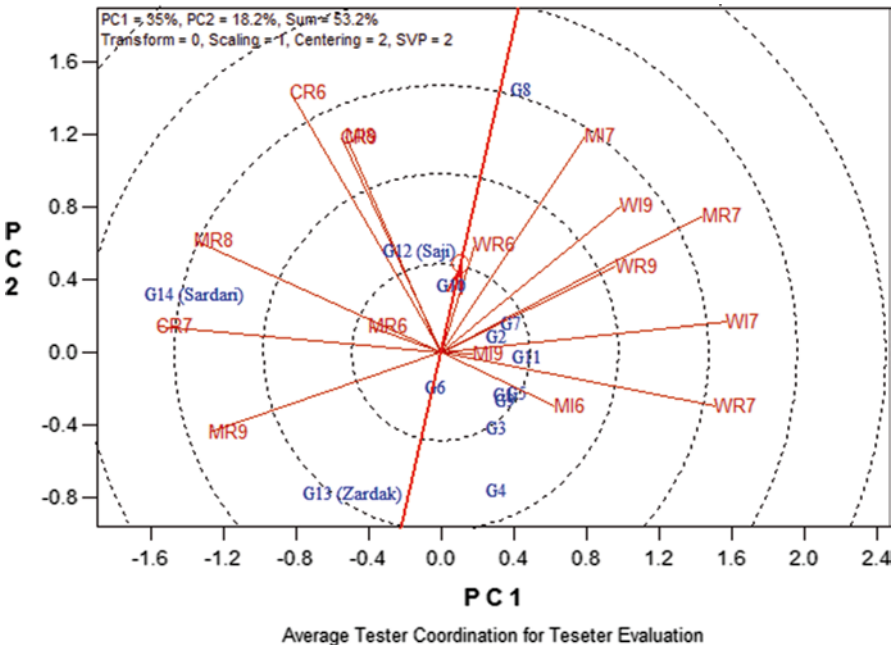

Fig. 14.8 GGE biplot which shows the discriminativeness vs representativeness ability of environments. The letters M, W and C included in the initial of environmental names stand for moderate, warm and cold locations, respectively; the letters R and I included in the environmental names stand for rainfed and irrigated conditions, respectively; and the numbers 6, 7, 8 and 9 included in the environmental names stand for 2005–2006, 2006–2007, 2007–2008 and 2008–2009 cropping seasons, respectively. G1–G14 are genotypic codes; G1–G11 breeding lines; G12: new cultivar; G13: old durum variety; G14: bread wheat old variety

tiveness and representativeness, the environment MI7 was identified as the most ideal environment.

Figure 14.8 also can be used to indicate test-environment representativeness of the mega-environment. Since the AEC abscissa is the *average-environment coordinate*, test environments that have small angles with the AEC are more representative of the mega-environment than those that have larger angles with the AEC (Yan et al. 2007). Based on this, the test environments can be classified into three groups. Group I are those that have short vectors and provide little or no information about the genotypes and, therefore, should not be used as test environments. Test environments MI9, MR6, WR6 and MI6 can be assigned to this group. Group II environments have long vectors and small angles with the AEC abscissa and are ideal for selecting superior genotypes. The best environments based on this group were MI7, MR8, CR9, CR6, WI9 and MR7. If budgetary constraints allow only a few test environments, group II test environments are the first choice (Yan et al. 2007). Group III environments have long vectors and large angles with the AEC abscissa. The environments CR7, WR7, MR8 and WI7 can be included in this group. These

environments cannot be used for selecting superior genotypes, but are useful in culling unstable genotypes.

According to Fig. 14.8, the maximum angle between the environments corresponding to cold location was well below 90°, showing the environments (CR7, CR6 and CR9) representing cold locations are correlated in ranking of the tested genotypes, which confirms the results by AMMI-2 biplot (Fig. 14.4). Similarly, the maximum angle between the environments (WR7, WI7, WR9, WI9 and WR6) corresponding to warm location was well below 90°, indicating that they tend to rank genotypes in a similar fashion which is in accordance with the results from AMMI-2 biplot (Fig. 14.4). The environments corresponding to cold locations were not correlated with those corresponding to warm locations, showing these two locations are different in ranking genotypes. Based on the results, these two locations can be regarded as two different mega-environments. In contrast, the environments corresponding to moderate locations were not associated in ranking genotypes, but they were correlated with both cold and warm environments.

The presence of extensive crossover GE interaction in the MET data, suggests that a systematic effort is needed to screen genotypes across different environments to identify those that perform well across or within a specific set of environments. The presence of wide obtuse angles between environment vectors (Fig. 14.8), indicates strong negative correlations among the test environments suggesting existence of strong crossover GE interaction across some environments for grain yield (Yan and Tinker 2005). This indicates that genotypes performing better in one environment would be performing poorer in another environment. At the same time, closer relationships among other environments are indicative of non-existence of crossover GE interaction, suggesting that ranking of genotype does not change from location to location. A mixture of crossover and non-crossover types of GE interaction in MET data is of very common occurrence (Fan et al. 2007; Mohammadi and Amri 2011, 2013; Rao et al. 2011; Yan and Tinker 2005). However, the which-won-where pattern is the most attractive feature of the GGE biplot, which graphically addresses crossover GE interaction, mega-environment differentiation, specific adaptation etc. (Fan et al. 2007; Gauch and Zobel 1997; Yan and Tinker 2005; Yan et al. 2000).

Relationships Among Univariate and Multivariate Models The Spearmen (1904) rank correlation coefficients between multivariate statistical models (AMMI and GGE biplot) and univariate (parametric and non-parametric) statistical methods are given in Table 14.4. No significant relationship was found between GGE biplot stability ranks with stability ranks of each of dynamic and static stability parameters. In contrast, significantly positive correlations were found between AMMI stability ranks with the joint regression parameters, Tai parameters, σ^2, YSi, $S_1^{(i)}$, $S_2^{(i)}$, $S_3^{(i)}$, $S_6^{(i)}$ and RS, showing these parameters are generally similar to AMMI in ranking of stable genotypes. No significant relationship was found between the stability ranks of GGE biplot and AMMI ($r = 0.27$).

Spearman (1904) rank correlations among the six statistical methods based on yield-stability ranks are given in Table 14.5. According to yield-stability ranks, JRA and Tai models were closely associated ($r = 1.0$; $P < 0.01$) in ranking of genotypes

Table 14.4 The Spearman (1904) rank correlation coefficients between univariate and multivariate statistical models based on stability ranks

Statistical model	GGE	AMMI
YLD	0.37	0.15
b	0.32	0.70**
S^2di	0.26	0.92**
α	0.31	0.71**
λ	0.23	0.87**
σ^2	0.28	0.95**
S^2x	–0.49	0.05
GAI	0.14	0.03
Pi	0.23	0.34
YSi	0.19	0.69**
$S_1^{(i)}$	0.35	0.79**
$S_2^{(i)}$	0.37	0.86**
$S_3^{(i)}$	0.32	0.80**
$S_6^{(i)}$	0.08	0.60*
TOP	0.15	–0.21
RS	0.32	0.65*
GGE	–	0.27
AMMI	0.27	–

YLD mean yield, *b* regression coefficient, S^2di variance in regression deviation, α alpha, λ lambda, σ^2 the Shukla (1972) stability variance, S^2x environmental variance, *D* AMMI statistic coefficient, *GAI* geometric stability index, *Pi* superiority index, $S_1^{(i)}$, $S_2^{(i)}$, $S_3^{(i)}$, $S_6^{(i)}$ the Huehn (1996) non-parametric stability statistics, *TOP* Fox et al. (1990) non-parametric statistic, *RS* the Kang (1988) rank sum, *YSi* yield-stability index, *GGE* genotype plus genotype x environment interaction, *AMMI* additive mean effects and multiplicative interaction

*, **Significant at 5 % and 1 % level of probability

for integrating yield with stability performance, indicating that they can be used interchangeably in crop breeding programs. Similarly, highly significant correlations were observed between YSi statistic, RS and AMMI model ($P<0.01$) and can be used simultaneously for selection for yield and stability performances. No significant relationship was found between GGE biplot and the other statistical methods in ranking of genotypes for integrating yield with stability performance. No relationship was observed between the regression models (JRA and Tai) with AMMI and Kang's parameters (RS and YSi). Although the GGE biplot was not significantly associated with each of the other methods, they generally gave similar results in identifying the most desirable and undesirable genotypes.

Rank correlations among the statistical methods also showed that the stability ranks given by the JRA (Eberhart and Russell 1966; Finlay and Wilkinson 1963), the Tai (1971) stability model and the Huehn (1979) non-parametric phenotypic

Table 14.5 The Spearman (1904) rank correlation between univariate and multivariate statistical models based on yield-stability ranks

	Parametric methods		Non-parametric methods		Multivariate methods	
Methods	JRA	Tai	RS	YSI	AMMI	GGE
JRA	1					
Tai	1.00**	1				
RS	0.33	0.32	1			
YSI	0.39	0.37	0.94**	1		
AMMI	0.44	0.42	0.95**	0.95**	1	
GGE	0.22	0.22	0.40	0.37	0.38	1

JRA joint regression analysis model, Tai (1971) stability model, *RS* the Kang (1988) rank sum, *YSi* yield-stability index, *AMMI* additive mean effects and multiplicative interaction, *GGE* genotype plus genotype x environment interaction
**Significant at 1 % level of probability

stability measures were positively associated with stability ranks given by AMMI model indicating that most of the univariate stability methods are highly correlated ($P<0.01$) with multivariate models i.e. AMMI in ranking of stable genotypes. The lack of relationship between stability ranks given by the univariate stability methods with stability ranks given by GGE biplot may be due relatively to low variance explained by GGE biplot (53.2 % of total variation). According to Yang et al. (2009) if the first two PCs explain more than 60 % of the (G + GE) variability in the data, and the combined (G + GE) effect account for more than 10 % of the total variability, then the biplot adequately approximates the variability in GE interaction data. In our study, the first two PCs explained less than 60 % of the variability (Fig. 14.5); and G and GE together accounted for less than 10 % of total variability (Table 14.1). Thus the biplots may not safely be interpreted as effective graphical representation of the variability in the MET data. Based on this, the results of biplot should be interpreted with caution (Yang et al. 2009).

The weaknesses of GGE biplot in analyzing genetic data may be revealed by the proportion of the total variation explained by the PC1 and PC2 of the biplot view. Depending on the complexity in the genetics of the trait under consideration plus the confounding effect of the environments, the biplot view may account for small variation, which makes the results of the analysis less worthy. Badu-Apraku et al. (2011) reported that the biplot generally accounted for small variation under stress environments, especially where the genotypes used were not specifically bred for tolerance to such stress. However, no studies have been carried out to specify when the proportion of variation explained by a biplot becomes too small to make a valid conclusion. However, it is generally assumed that any proportion below 40 % is too small (Akinwale et al. 2014).

Although no significant relationships were found between GGE stability ranks with univariate stability ranks and AMMI stability ranks, they generally identify the most desirable and undesirable genotypes. For example and based on GGE biplot,

AMMI and univariate stability methods, the genotypes G6, G10 and G12 were stable and G14 and G13 landraces were unstable. The results demonstrated that the multivariate models and univariate stability methods generally gave similar results in indentifying the most desirable and undesirable stable genotypes. However, the results showed that although the variation explained by the methods is not the same, they generally also give similar results in identifying the most desirable and most undesirable genotypes for integrating yield with stability performance. This can be confirmed by consistent results obtained by the models in characterizing G14 and G13 as low yielding and unstable entries and, G12, G11, G10 and G9 as high yielding and stable lines. Several comparative studies have been performed between GGE biplot and JRA in maize (Alwala et al. 2010) and triticale (Goyal et al. 2011); between JRA and AMMI models in cereal crops (Annicchiarico 1997) for stability analysis; between GGE biplot and AMMI in different crops (Gauch 2006; Gauch et al. 2008; Yan et al. 2007) and between GGE biplot and non-parametric methods for stability analysis in durum wheat (Mohammadi and Amri 2012). However, in comparison with univariate models, the multivariate models are more efficient in analyzing GE interaction, because they can provide the biplots and information on genotype, environment and their interaction, while the univariate methods give information only on genotype evaluation. However, if a breeder would like to evaluate the genotypes across diverse environments, the univariate methods can be a good alternative for some options of multivariate models which are related to genotype evaluation.

Genotype by Trait (GT) Biplot Analysis The polygon view of a GT biplot is the best way to visualize the interaction patterns between genotypes and traits and to effectively interpret the results of the biplot (Yan and Rajcan 2002). Figure 14.9 presents a GT biplot with a polygon view representing the data of 14 entries and 10 agronomic traits across environments. The biplot based on multiple traits explained 49.8 % of the total variation of the standardized data. According to Fig. 14.9, the ten traits studied fell into three groups with top characterized genotypes based on each group. The phenological traits i.e. days to heading (DH) and maturity (DM) along with flag-leaf length (FL), peduncle length (PL), spike length (SL) and plant height (PH) tend to be separated from the other traits with the genotype G13 (durum landrace) being the latest in flowering and maturity and tallest in stature. The second group consisted of the grain yield (YLD), biological yield (Bio) and harvest index (HI) with G4 being the best genotype. The 1000-kernel weight (TKW) formed the next group with G10 having high value.

Figure 14.10 represents a GT biplot showing the relationships among studied traits across environments and captures 49.8 % of the information in the standardized data of the 14 entries for the 10 traits studied. This average percentage reflects the complexity of the relationships among the measured traits. According to Kroonenberg (1995), the fundamental patterns among the traits should be captured by the biplots. The traits PL, FL and PH were strongly correlated in discriminating the genotype G13, followed by G3, G9 and G14. These three traits were not associated with TKW. The best genotypes based on TKW were G10, G8 and G2. The YLD,

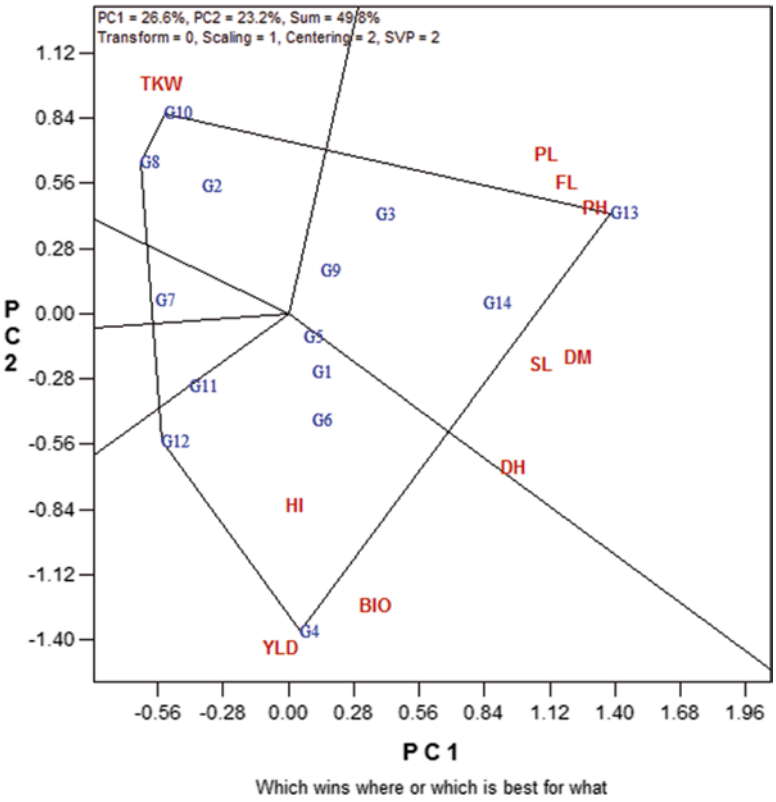

Fig. 14.9 *Which is best for what* view of genotype by trait (GT) biplot of 14 genotypes (G1–G14) for 10 agronomic traits across environments (combination of 2 years and 2 rainfed and irrigated conditions). *DH* days to heading, *DM* days to maturity, *PH* plant height, *SL* spike length, *PL* peduncle length, *FL* flag-leaf length, *Bio* biomass yield, *TKW* thousand kernel weight, *HI* harvest index, *YLD* grain yield. G1–G14 are genotypic codes; G1–G11 breeding lines; G12: new cultivar; G13: old durum variety; G14: bread wheat old variety

biological yield and HI due to their acute angles tend to discriminate the genotypes G4, G6 and G12 and diversely differed from TKW in ranking of genotypes. The DM, SL and DH were related to each other in ranking of genotypes and the genotypes with high TKW can be discarded when using these three traits (DM, SL and DH). The genotypes G7, G11 and G12 had a good combination of YLD and TKW and due to their positions which are placed in opposite direction of DH and DM in the biplot tend to flower and mature before the other genotypes (Fig. 14.10). The genotypes G5, G9 and G1 which were near to the origin of the biplot had average performance based on the multiple traits. An important advantage of the GT biplot is that it can be used to identify redundant traits in an effort to reduce cost in measuring traits in field experiments without sacrificing precision. Therefore, the high positive correlation between PL, FL and PH suggests one (i.e. PH) of these

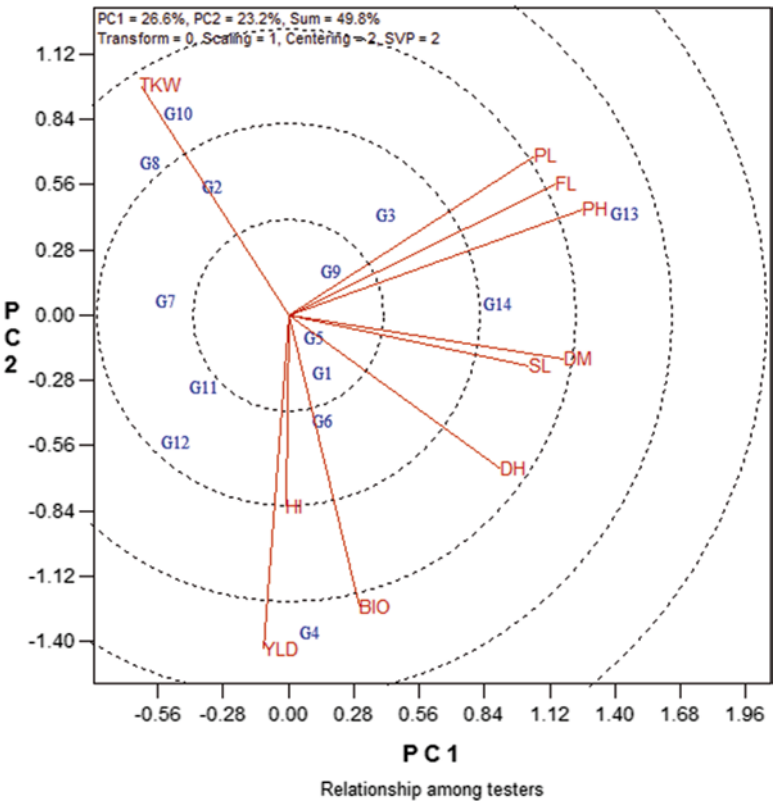

Fig. 14.10 GT biplot showing relationship among traits across environments (combination of 2 years and two rainfed and irrigated conditions). *DH* days to heading, *DM* days to maturity, *PH* plant height, *SL* spike length, *PL* peduncle length, *FL* flag-leaf length, *Bio* biomass yield, *TKW* thousand kernel weight, *HI* harvest index, *YLD* grain yield. G1–G14 are genotypic codes; G1–G11 breeding lines; G12: new cultivar; G13: old durum variety; G14: bread wheat old variety

traits will be sufficient as a selection criterion. Similarly, the high correlation between YLD, Bio and HI suggests that one (i.e. YLD) of these traits will be sufficient as a selection criterion. The correlation between the DH, DM and SL also suggests one (i.e. DH) could be used as selection in durum breeding program.

The distance between the genotype and the biplot origin, is a measure of genotype peculiarity (i.e., how it differs from an *average* genotype), which is a hypothetical genotype that has an average level for all traits represented by the biplot origin (Yan and Frégeau-Reid 2008). Therefore, genotypes with long distance are those that have extreme levels for one or more traits. Such genotypes may or may not be superior in yield but they may be useful as parents for some useful traits. Based on the distance of genotypes in Fig. 14.10, the tested genotypes were arbitrarily stratified into two groups. The first group consists of G4, G13, G10, G8, G12

and G14 entries with vectors longer than 50 % of the longest genotype vector (i.e. G13) and the second group formed of the rest of entries with vectors shorter than 50 % of the longest genotype vector. This confirms that the GT biplot can provide a quick visual mean for identifying genotypes that have extreme and useful trait profiles (Yan and Frégeau-Reid 2008).

The results also indicated that the genotype performances with respect to different traits in one environment differed from genotype performances in the other environments, reflecting genotype-by-trait-by-environment interaction. However, these interactions complicate the understanding of genotype-by-trait analysis in a breeding program. The biplot analysis of genotype by trait data helped in finding durum genotypes for future breeding programs. The results identified parental germplasm for earliness, short stature, high grain weight and yield, which can be used in durum wheat breeding in Iran. By applying the GT biplot technique to the durum wheat MET data, in comparison to the Peterson et al. (2005) correlation coefficients, interrelationships among traits were clearly shown, providing more information on these relationships than other commonly used methods, such as path coefficient analysis (Rubio et al. 2004). Similar reports demonstrated that the GT biplots were an excellent tool for visualizing genotype-by-trait data and revealing the interrelationships among traits (Egesi et al. 2007; Fernandez-Aparicio et al. 2009; Peterson et al. 2005; Yan and Kang 2003).

However, there are several considerations related to the significant improvement of yield and stability performance. Modern cultivar Saji and the breeding lines G8 and G10 which combine high yields and stability are outstanding genotypes which can be used in breeding programs. Significant improvements obtained in warm and moderate locations were increased when supplemental irrigation was applied. The old varieties were characterized by a minimal responsiveness to improved environmental conditions, while the breeding lines were highly responsive to favorable environments and showed a pronounced adaptation. Results from the present study confirm the little significant yield gains in cold areas during the last decade, as already reported in previous studies (Mohammadi et al. 2010a, 2011). The results of stability showed that the high grain yield and stability analysis are not mutually exclusive, which are supported by other researchers (Morgounov et al. 2010; Ortiz et al. 2001; Tollenaar and Lee 2002). The results from this study suggest that breeding lines combine both improved yield and improved yield stability. Positive genetic gains in warm and moderate locations compared to cold location suggests the importance of evaluation of the breeding materials in warm and moderate conditions. If there are plans to expand the cultivation of durum wheat to the highlands of Iran and other areas with cold winters, special efforts are needed to develop durum wheat germplasm with good cold tolerance and winter hardiness (Mohammadi et al. 2014). This approach will need to use the old varieties and available parental germplasm developed for cold areas in Europe. In addition, introgression of these cold tolerance traits can also be done from wild *Triticum* and *Aegilops* species which

exhibit a winter growth habit. Successful genotypes of durum wheat need to be adapted to a broad range of environmental conditions in Iran in order to ensure their yield stability and economic profitability. Farmers are more interested in cultivars that produce consistently higher yields under their growing conditions and breeders also want to fulfill these needs. Hence, the information on GE interaction and stability is of paramount importance for wheat breeders and farmers. In summary, selection of genotypes for stability is needed under rainfed conditions, where the environment is variable and unpredictable. Therefore, genotypic evaluation under variable environments and adoption of simultaneous selection for yield stability performance and tolerance to different abiotic stresses is the most valuable selection index to lead to desirable durum wheat varieties.

Although selecting specific genotypes for specific environments is the best way to utilize GE interactions, it may not be practicable. GE interactions usually cannot be related to a single or even a few environmental factors, thus it might not be possible to group cultivation environments into groups which give the same GE response. If the environments in the target region can be grouped, then limited resources for plant breeding may dictate that stable genotypes with wide applicability are the best option. Because good performance is the main objective of plant breeding, simultaneous selection for yield and stability of performance is an important consideration in breeding programs (Kang 2002; Kang and Magari 1996). To anticipate future needs in facing climate changes in the region, it might be worthwhile to target improved yield stability of new cultivars. In this study, durum wheat breeding lines showed high yield and better yield stability than the control cultivars. However, in the long term, breeding and selection for yield potential might reduce yield stability reported from many studies (Calderini and Slafer 1998; Loomis and Connor 1996). The development of high and stable yielding germplasm will be highly desired by the breeder to face the adverse effects of climate change. To increase the stability of crops, growing genotypes under stressful environmental conditions (i.e. drought, cold and warm stresses) will help breeders in identifying outstanding candidate genotypes to enhance both yield and stability performance in breeding programs. Insufficient yield stability in crop cultivars has been recognized as one of the main factors responsible for the gap between yield potential and actual yield, particularly in drought-prone environments. Crop improvement efforts have benefited greatly from advances in available data, computing technology and methods for targeting genotypes to environments. These advances support the analysis of GE interaction to understand how well a genotype adapts to environmental conditions. Rainfall and temperature are among the environmental factors which are often unpredictable in Mediterranean environments, and result in inconsistent environmental conditions for crop growth and a critical source of uncertainty for farmers and growers. To overcome the influence of GE interaction on yield production it can be exploited by selecting for specific adaptation, or minimized by selecting for wide adaptation.

14.4 Conclusions and Prospects

The presence of extensive crossover GE interaction clearly suggests that efforts are necessary to exploit GE interaction by specific adaptation or to minimize GE interaction by wide adaptation. The results obtained showed the potential of genetic material for identifying promising genotypes for each of exploitation and minimization strategies in durum wheat breeding program.

Applied statistical procedures have clearly and conveniently aided in the identification of stable and superior high yielding genotypes in variable environments. Significant positive correlations between parametric and non-parametric stability procedures indicate that they can be used interchangeably for assessing the genotypes for both stability and for integrating yield with stability performance in crop breeding programs. The parametric stability methods have good properties under certain statistical assumptions, like normal distribution of errors and interaction effects; however, they may not perform well if these assumptions are violated. However, when the data do not met normal distribution assumptions, using non-parametric stability measures instead of parametric ones can be recommended, because non-parametric stability measures are not generally affected by data distribution.

The results presented demonstrated the advantages of the multivariate models compared to univariate stability models for analyzing GE interaction in durum MET data. Simultaneous assessment of genotypes and environments in biplot facilitates the interpretation and identification of specific interactions among them. The multivariate models allowed a meaningful and useful summary of GE interaction data and assisted in examining the natural relationships and variations in genotype performance among various testing environments. Although the AMMI and GGE biplot gave generally similar results in identifying superior genotypes and in characterizing the desirable test site, the GGE biplot was more versatile and flexible, and provided a better understanding of GE interaction than the AMMI method.

The genotype by trait biplot is a useful tool for exploring multiple trait data, trait profiles of the genotypes and can help in the determination of contrasting genotypes based on the trait(s) for improving genetic materials in durum wheat breeding program. The results identified parental germplasm for earliness, short stature, high grain weight and yield, which can be exploited in durum wheat breeding program.

A positive increase in yield and yield stability is attributable predominately to genetic improvement of durum breeding lines. Increased yield resulted from the trend in durum breeding programs to test and develop durum breeding germplasm for wide adaptation, which has increased yield stability. The yield stability of the high-yielding breeding lines was variable but there were few genotypes combining both yield stability and high yield, indicating genetic improvement for both high yield and stability performances in durum breeding lines under rainfed areas of Iran.

Introduction of an effective package of statistical models for exploring all information existing in GE interaction of MET data is necessary in a breeding program. Based on the results given, in regard to ranks given by different statistical model and

correlation matrix analysis, and to avoid any decision error on genotype recommendation by breeder/agronomist, we suggest to incorporate both parametric (i.e. joint regression analysis or the Tai stability model) and non-parametric (i.e. YSi statistic) univariate analysis procedures with the most appropriate multivariate analysis models (i.e. GGE biplot) due to existence some pitfalls in interpreting the biplots when the biplot does not fully approximate the data. Therefore by using this package the risk of genotype recommendation and breeding decisions by breeders for unpredictable environmental conditions in a crop breeding program will be considerably reduced.

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Part IV
Biotic Stress Resistance

Chapter 15
Breeding Strategies for Improving Plant Resistance to Diseases

Thomas Miedaner

Abstract Durable disease resistance is an important aim in each breeding program. Genetically, two basic patterns of resistance are available: qualitative (race-specific, vertical) and quantitative (race-non-specific, horizontal) resistances. Classical breeding methods are recurrent backcrossing (BC) for introducing single (major) genes, recurrent selection for improving the level of quantitative resistances and multi-stage selection for combining resistances and agronomic traits during cultivar development. Molecular markers allow efficient introduction of qualitative resistances into elite material and to analyze quantitative resistances. During marker-assisted backcrossing (MABC), a major gene can be precisely targeted, the genome of the recurrent parent can be recovered fast, and linkage drag can be reduced. By marker-assisted selection (MAS), major genes or quantitative trait loci (QTL) can be pyramided. Genomic selection (GS) will allow selecting for multiple traits directly in the genome by chip-based, high-throughput genotyping platforms. To achieve a higher durability, populations of biotrophic pathogens (e.g. powdery mildews, rusts) should be regularly monitored for their virulence frequencies and virulence combinations. Strategies for enhancing durability of qualitative resistances aim to increase host diversity or host complexity. Quantitative resistances generally have a higher durability but might be prone to gradual loss (erosion) in the long term. Limits of resistance selection are given by several biological and economic constraints. Broad-spectrum resistance genes and GS might open new avenues to a rational, knowledge-based selection. Resistance breeding will remain a top priority given the challenges of a growing world population in a changing climate.

Keywords Backcross • Genomic selection • Marker-assisted breeding • Pathogen monitoring • Qualitative resistance • Quantitative resistance

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15.1 Introduction

Disease resistance is an important trait in every breeding program. The commercial significance depends on the crop, losses caused by the disease, alternative measures for disease control, availability of resistance sources and the ease of selection. Moreover, several pathosystems can only be controlled by disease resistance. This includes most virus and nematode diseases, but also some fungal diseases, like ergot in cereals (*Claviceps purpurea*), *Rhizoctonia solani* in maize and sugar beet, powdery scab (*Spongospora subterranea*) in potato or black leg (*Plasmodiophora brassicae*) in rapeseed.

The vulnerability of a crop to diseases depends mainly on its genetic structure (Table 15.1) Line cultivars (e.g. wheat, barley, oats, peas) with plants being homozygous at all loci and having a homogeneous phenotype, are prone to diseases. The same holds true for asexually propagated clonal cultivars (potato, strawberry, banana, fruit trees) resulting in highly uniform stands. The vegetative propagation unit (tuber, bulb, cutting) enables more pathogens to survive across years than by sexual propagation via seeds. Single-cross hybrids are also homogeneous due to the controlled crossing of two inbred lines. Three-way and double-cross hybrids are segregating, resulting in a similar genetic structure like population cultivars. The latter have a high buffering capacity, because they are heterogeneous and the majority of loci is heterozygous. Most crops in industrial countries are, however, genetically uniform and, therefore, prone to disease epidemics.

In each crop there are must-have disease resistances, mostly to viral diseases where no alternative control measure exists or to diseases leading to mycotoxin contamination, and nice-to-have resistances that can also be controlled by pesticides. For the latter diseases, it might be enough to eradicate the most susceptible progenies in a breeding program. In whatever way, resistance breeding is an environmentally friendly and for the farmer most cost-effective way of crop protection.

Table 15.1 Reproductive system, type of cultivar and genetic structure of the cultivar

Reproductive system	Type of cultivar	Genetic structure (Genotype/phenotype)	Vulnerability
Sexual:			
Self-pollination	Line cultivar	Homozygous/homogeneous	High
Cross-pollination	Population cultivar	Heterozygous/heterogeneous	Low
Controlled crossing	Hybrid cultivar	Heterozygous/homogeneous[a]	High
Asexual:			
Vegetative	Clonal cultivar	Heterozygous/homogeneous	High

Source: Schnell (1982)

[a]Assuming a single-cross hybrid

This chapter concentrates on classical breeding strategies, those including DNA markers with a focus on small-grain cereals and a short section on transgenic approaches. For detailed information on molecular breeding and genomics please refer to the respective chapters in this book. Resistance breeding approaches to maize and rice diseases are reviewed in other chapters in this book.

15.2 Genetics of Disease Resistance

Van der Plank (1963, 1968) was the first to categorize resistances as either vertical or horizontal (Table 15.2) and correspondingly the degree of pathogenicity in the pathogen as virulence and aggressiveness, respectively (see Table 15.3). Unfortunately, terminology is used inconsistently and many terms exist for each type of resistance that are not necessarily synonymous. In this review, the terms qualitative and quantitative resistance are used throughout.

Comparing many plant pathosystems, the simple black-and-white scheme from Table 15.2 is becoming gray (Poland et al. 2009). Generally, race-specific resistance is governed by major genes resulting in a hypersensitive response; they are often associated with complete resistance, and are highly effective through the entire life cycle of the host plants (=all-stage resistance), but of low durability. Some monogenic resistances, however, are not expressed through hypersensitivity, are race-non-specific, show a partial degree of resistance, an adult-plant expression and have a high durability. Examples are *Lr34* (wheat-rusts), *Yr36* and *Yr39* (wheat-stripe rust). A few other genes expressing complete resistance (e.g. *mlo*) are nevertheless durable. Quantitative resistances are often called adult-plant resistances (APR), because they either express susceptibility at the seedling stage (e.g., powdery mildew, rusts) or the resistances in both stages do not correspond (e.g., *Fusarium*

Table 15.2 Most commonly observed characteristics of qualitative and quantitative resistance

Category	Qualitative resistance	Quantitative resistance
Synonyms	Vertical, differential	Horizontal, uniform, general
Pathogen specify	Race-specific	Race-non-specific
Symptoms	No disease	Varying degree of disease
Degree of resistance	Complete, absolute	Incomplete, partial
Mechanism	Hypersensitivity	Diverse
Plant growth stage	All-stage resistance (seedling resistance)	Different in each stage (adult-plant resistance, APR)
Assessment	Infection type	Disease severity
Durability	Low	High
Inheritance	Mono-, digenic	Oligo-, polygenic
Gene effect	Major	Minor
Breeding strategy	Backcross breeding	Multi-stage/recurrent selection

Table 15.3 Definitions of important terms for disease resistance

Term	Definition
Adult-plant resistance	Resistance only visible in the adult stage of a plant, i.e. at the generative phase. Adult-plant resistance can be inherited monogenically or quantitatively and need not to be durable
Aggressiveness	Degree of pathogenicity in a quantitative host-pathogen interaction; it varies quantitatively from low to highly aggressive indicating a low to high damage of the host
Avirulence (gene)	A gene (*Avr*) in a pathogen that causes the pathogen to elicit an incompatible (defense) response in a resistant host plant. Interaction of an avirulence gene product with its corresponding plant resistance (R) gene is highly specific and usually provokes a hypersensitive reaction
Broad-spectrum resistance locus	Individual locus that confers resistance to multiple races of a pathogen species or multiple taxa of pathogens
Durable resistance	Resistance that remains effective for a long period when applied on a large scale in a region that is undergoing regular epidemics of the pathogen (Johnson 1981)
Epistasis	Interaction between genes at different loci
Pathogenicity	Ability of an (micro)organism to damage a healthy plant
Pathotype	Isolate with a special combination of avirulences/virulences
Pathosystem	Combination of a specific host and pathogen species or a complex of closely related pathogen species
Quantitative trait locus (QTL)	Markers linked to the genes that underlie a quantitative trait; it should be remembered that there is only a genetic linkage between markers and genes based on recombination frequencies
Race	Isolates within a pathogen species that are distinguishable by their virulence, but not by morphology. Today, races are often a complex combination of virulences, thus *pathotype* might be the better term
Qualitative resistance	Race-specific resistance inherited by single R genes, also named vertical resistance or hypersensitivity resistance following the gene-for-gene concept
Quantitative resistance	Resistance inherited by several genes with minor effects, usually non-race-specific and prone to non-genetic interactions, also named horizontal resistance
Virulence	Degree of pathogenicity in a qualitative host-pathogen interaction; low virulence indicates a virulence to a few R genes, high virulence to many R genes

Sources: (Miedaner and Korzun 2012; Niks et al. 2011; van der Plank 1963, 1968)

diseases). But adult-plant resistance can also be inherited monogenically, e.g. for some leaf rust (*Lr*) or stripe rust (*Yr*) resistance genes. This demonstrates that a few qualitative resistances cannot be detected at the seedling stage. The lifestyle of a pathogen has no direct association to the type of resistance. Biotrophic pathogens generally provoke qualitative resistances, but all of them can also be controlled by quantitative resistances; both types may even coexist in the same cultivar (Miedaner and Flath 2007). For necrotrophic pathogens generally quantitative resistances apply, however, some necrotrophs (e.g. *Zymoseptoria tritici*) are controlled additionally by monogenically inherited, isolate-specific genes. A long co-evolution

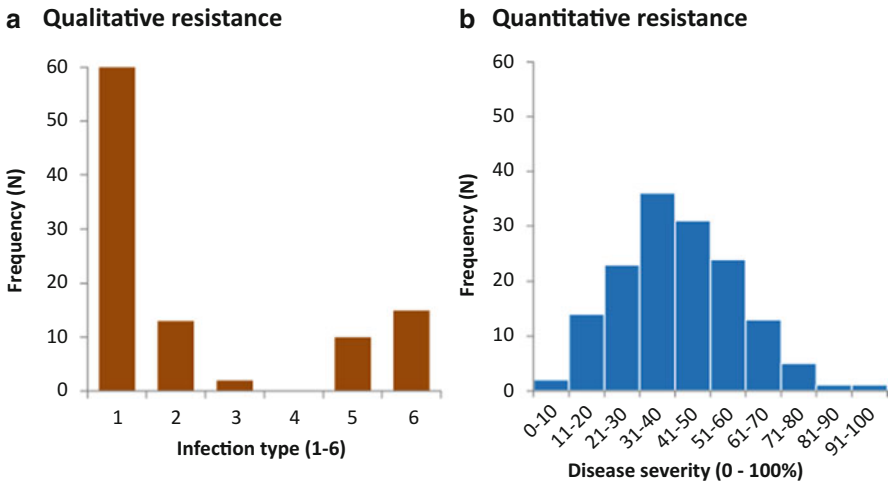

Fig. 15.1 Qualitative and quantitative resistance in segregating progenies after a cross resistant x susceptible. **a** Resistance is inherited monogenically dominant, segregation of infection type follows 3:1 ratio (e.g. wheat/leaf rust). **b** Resistance is inherited oligo-/polygenically, disease severity follows a normal distribution (e.g. wheat/*Fusarium* head blight)

between host and pathogen might lead to a larger variation in resistance types. Because of much confusion in terminology we provide here a short glossary of important terms for disease resistance (Table 15.3).

Genetics of resistances can be analyzed by crossing a resistant with a susceptible parent and evaluating the F_2 single plants, lines in higher selfing generations or doubled-haploid populations (Fig. 15.1). For the breeder, it is most important to differentiate between resistances that are segregating qualitatively, i.e. producing distinct classes assessed as infection type, or quantitatively, i.e. revealing a continuous distribution assessed as disease severity (Fig. 15.2a–c). Typically, qualitative traits are subjected to chi-square tests of expected Mendelian segregation ratios while quantitative traits are subjected to analyses of variance (ANOVA). This categorization applies, however, only for experiments with high heritability (proportion of genotypic to phenotypic variance) because a monogenic trait also can provide a continuous phenotypic distribution when environmental and/or error effects are high (Allard 1960).

Inheritance of disease resistance is a complex topic including methods of plant breeding, quantitative genetics and molecular biology that cannot be fully covered by this review; see: (Keller et al. 2000; Niks et al. 2011).

15.2.1 Qualitative Resistances

Monogenic resistances are usually caused by a hypersensitive response (HR). Rapid death of invaded host cells is induced after the gene product of a pathogen encoded by an avirulence (*Avr1*) gene is recognized by a gene product from the plant encoded

by a corresponding resistance gene (*R1*), leading to an incompatible reaction (=resistance, Fig. 15.3a). If the plant has only susceptible alleles at this locus (*r1*), the reaction is always compatible (susceptible) independently of the genotype of the pathogen. Likewise, if the pathogen is virulent for *R1* (*avr1*), all reactions are compatible. These patterns are described by the gene-for-gene hypothesis (Flor 1956, 1971) indicating that each resistance gene in the plant has a matching avirulence gene in the pathogen. Flor was the first who analyzed the inheritance of resistance in the host (flax) and avirulence in the pathogen (flax rust, *Melampsora linii*) simultaneously. Since then, this *hypothesis* has been verified in many plant-pathogen interactions with a qualitative inheritance of resistance. If the resistance gene is dominantly inherited, the scheme can be simplified to result in the well-known *quadratic check*, because the presence of one resistance allele is enough to promote resistance (Fig. 15.3b).

Most qualitative resistance genes (R genes) involved in the resistances to fungi and viruses belong to the largest class of R genes with a nucleotide-binding site plus leucine-rich repeat (NB-LRR) (Keller et al. 2000). Fast production of oxidants is a typical indicator for HR. R and Avr genes are mostly inherited dominantly although in some virus resistances also recessive R genes are involved (Ordon et al. 2004). Race specificity, assessed by confronting host genotypes by pathogen races on the basis of this gene-for-gene concept, is usually a strong hint for qualitative resistances, but seedling or leaf-segment tests apply only to all-stage resistances.

Many pathogen populations are notorious for their rapid adaptation to R genes. They form new virulent (*avr*) pathotypes by mutation of the *Avr* gene and, thus, avoid host recognition and host-resistance reactions. Virulent races rapidly attack previously resistant cultivars. This is often called *breakdown of resistance*, but in fact the pathogen made the R gene ineffective by mutation to virulence. The virulent pathotypes can rapidly spread in pathogen populations when the mutation has no negative effect on the fitness of the pathogen. Gene-for-gene relationships have been identified in many plant-pathogen interactions, including bacteria, fungi, nematodes, viruses and insects. Mostly, biotrophs are included, like rusts (*Puccinia* spp.), powdery mildew (*Blumeria graminis*), smuts (*Ustilago* spp.), bunts (*Tilletia* spp.), potato blight (*Phytophthora infestans*), but also some necrotrophs, like rice blast (*Magnaporthe grisea*), septoria tritici blotch (*Zymoseptoria tritici*) or northern corn leaf blight (*Setosphaeria turcica*).

Resistance of most R genes can already be assessed at seedling stage because the genes are active through the whole lifecycle of a plant (=all-stage resistance). This is easily done by a seedling or a leaf-segment test (=detached leaf test) in the greenhouse or laboratory. The first and/or second leaf is inoculated by distinct races and the reaction of the plant is rated after 10–14 days. For rating, the infection type is used, i.e. a description of the host response symptoms (Table 15.4).

Cultivars relying solely on race-specific genes may become susceptible within a few years (Bayles et al. 2000; Chen and Line 1995; Kolmer 2013). Consequently, serious yield losses can occur. Typically, many R genes are available in each patho-

Fig. 15.2 Qualitative resistance for stem rust in winter rye. **a** Complete resistance and **b** complete susceptibility. **c** Quantitative resistance for leaf rust in winter rye varying from low to highly susceptible

system. For rusts in wheat, currently about 70 formally and 11 temporarily designated genes for leaf rust (*Lr*) caused by *Puccinia triticina*, 58 genes for stem rust (*Sr*) caused by *P. graminis* and at least 53 formally and 39 temporarily designated genes for yellow rust (*Yr*) caused by *P. striiformis* have been described (McIntosh et al. 2012), most of them are race-specific. The high resistance level, simple inheritance and easy incorporation into commercial cultivars make them attractive to breeders (Fig. 15.2a, b) (Bolton et al. 2008; Chen 2007; Hovmøller 2007). When deploying qualitative resistances in commercial cultivars the race composition of the target

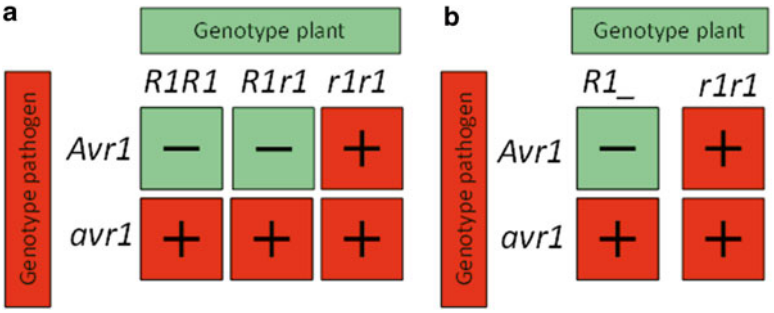

Fig. 15.3 Explanation of the gene-for-gene interaction for a diploid plant with one dominant resistance gene (*R1*) and a haploid pathogen with avirulence (*Avr1*) and virulence (*avr1*); + denotes a compatible reaction (susceptibility), – an incompatible reaction (resistance). **a** Full scheme with all possibilities; **b** quadratic check for dominantly inherited resistance genes

Table 15.4 Different rating scales of infection type for cereal rusts (numerical scale after McNeal et al. 1971; descriptive scale after McIntosh et al. 1995)

Infection type		Description	Resistance class
Numerical	Descriptive scale		
0	0	No visible signs or symptoms;	Immune
1	;	No sporulation; necrotic and/or chlorotic hypersensitivity flecks;	Highly resistant (HR)
2		No sporulation; necrotic and/or chlorotic blotches or stripes	Resistant (R)
3	1	Trace sporulation; necrotic and/or chlorotic blotches or stripes	Moderately resistant (MR)
4		Light sporulation; necrotic and/or chlorotic blotches or stripes with	Intermediate types of resistance
5	2	Intermediate sporulation; necrotic and/or chlorotic blotches or stripes	
6		Moderate sporulation; necrotic and/or chlorotic blotches or stripes	
7	3	Abundant sporulation; necrotic and/or chlorotic blotches or stripes; chlorosis behind the sporulation area	Moderately susceptible (MS)
8		Abundant sporulation with necrotic and/or chlorotic blotches; light chlorosis behind the sporulation area	Susceptible (S)
9	4	Abundant sporulation with necrotic and/or chlorotic blotches; no chlorosis behind the sporulation area	Highly susceptible (HS)

region, however, must be monitored regularly by a differential set (see Sect. 15.6.1), because only those resistance genes will be effective where the corresponding virulence frequencies in the pathogen population are low or absent. Additionally, the matching isolates with known (a)virulences can be used by breeders to determine the occurrence of R genes in their breeding populations and to select for resistances mediated by still-effective genes. Only a few R genes remained effective over a long period (see Sect. 15.6.1).

For selecting qualitative adult-plant resistances, the easiest way is to grow breeding populations in as many field locations as possible and to rate which host genotypes are not or only low infected. As a check the same genotypes should be tested as susceptible in the seedling stage with an array of isolates. Growing the differential set in the same experiment will monitor the pathogen population at each location and shows which R genes are still effective.

15.2.2 Quantitative Resistances

Quantitative resistances are usually expressed partially (Fig. 15.2c) and offer an option towards higher durability. They exhibit reduced infection frequency, increased latent period as well as smaller and fewer spore deposits (Ohm and Shaner 1976; Parlevliet 1985). In some pathosystems, quantitative resistances can be expressed on such a high level that they nearly approach complete resistance (Fig. 15.5). Quantitative resistances are inherited by several genes that can interact with each other (epistasis) and with the environment (Kearsey and Pooni 1996). They are specific for plant growth stages and/or plant tissues. *Fusarium culmorum*, for example, can infect all cereal parts, but ranking of genotypes in their resistances to seedling blight, foot rot or head blight is different (Arseniuk et al. 1993; Miedaner 1997). In the barley/net blotch (*Pyrenophora teres* f. *teres*) and spot blotch (*Cochliobolus sativus*) pathosystems, resistances were controlled in seedling stage by different loci than in adult-plant stage as revealed by QTL analyses (Steffenson et al. 1996).

In practice, quantitative resistances are selected in the respective plant stage in the field by artificial inoculation across locations and years. Additionally, the time of rating is crucial. While a complete, qualitative resistance can just be rated at the end of the epidemic, for quantitative resistances an optimal time for genotypic differentiation exists (Fig. 15.4). The assessment can be done by area under disease progress curve (AUDPC) regarding all rating dates, the best single rating (Fig. 15.5) or an arithmetic mean of the dates with best genotypic differentiation.

Selection of quantitative resistances in the presence of qualitative resistances, like in cereal rusts and powdery mildew, is problematic (Parlevliet 1989). To avoid confounding effects with effective major genes segregating in the breeding population, a seedling test should be applied first. Screening either with all effective avirulence/virulence combinations present in the region or a highly virulent race would remove all major genes from the host population. Afterwards, progenies can

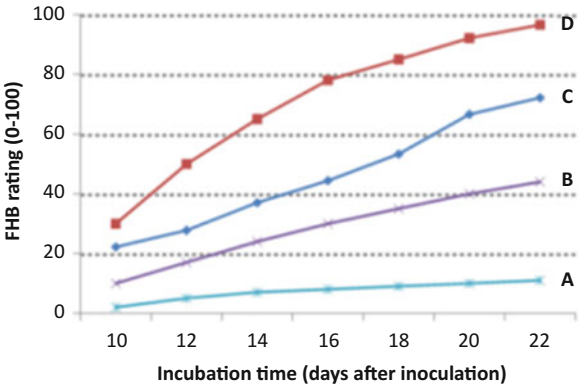

Fig. 15.4 Disease progress curves of four cultivars for wheat/Fusarium head blight (*FHB*). Cultivars *A*–*D* show different degrees of quantitative resistances from highly resistant (*A*) to highly susceptible (*D*)

Fig. 15.5 Four spring wheat lines with different levels of quantitative resistance to *Fusarium* head blight after inoculation with *Fusarium culmorum*; the two lines on the left are descendants of crosses with the highly resistant Chinese landrace Sumai 3, the two lines on the right are highly susceptible, commercial cultivars

be analyzed in the field for adult-plant resistance (e.g. Parlevliet 1995). It is, however, not possible to differentiate between quantitative and monogenic adult-plant resistance when no hypersensitivity response occurs in the latter.

Quantitative resistances are usually characterized to be race-non-specific. However, within the last decade evidence was collected that some QTLs are effective only against a subset of pathogen isolates. In the rice/*Pyricularia grisea* pathosystem, for example, only 2 out of 12 QTLs had an effect on all three tested isolates, the other had effects on only one or two isolates (Chen et al. 2003). Similar results

came from several other pathosystems (Poland et al. 2009; Sørensen et al. 2014) leading to the hypothesis that there might be three types of resistances: (1) basal (overall) resistance governed by many QTLs in the classical sense, i.e. race-non-specific, and largely conserved across host species and even pathogens (broad-spectrum QTLs); (2) quantitative resistance mediated by QTLs that are specific for a pathosystem and might be effective only against a subset of isolates; (3) qualitative, hypersensitivity-based R genes. It can be speculated whether QTLs of the type (2) are just *defeated* race-specific resistance genes with some residual effect (Young 1996). Poland et al. (2009) present six hypotheses for explaining the mechanisms underlying resistances of type (1) and (2).

In all pathosystems analyzed so far, quantitative resistances of varying degrees were detected. For breeding purposes, a quantitative assessment scale, standards with known resistance level, several locations and/or years (=environments) and perhaps genetically different isolates are indispensable for selection.

15.3 Resistance Sources

The availability of resistance sources is the most critical point when starting a breeding program (Table 15.5). The ideal scenario is when enough genetic variation is already available in known breeding populations or cultivars adapted to the target region. This can often be found for traditional pathosystems where resistance breeding has a long history, e.g. wheat/powdery mildew in Europe. Then, resistance sources have just to be tested for other agronomic traits and can directly be introduced into elite cultivars by crossing. It is similarly easy, although taking some more time, when resistance sources can be acquired from neighboring countries of the same climate zone, e.g. resistances for wheat/stem rust from Austria or Hungary for German cultivars. Here, phenological development and yield potential might be different, but this can be overcome by subsequent recurrent selection.

Much more effort is needed, when resistance sources are non-adapted cultivars, e.g. from Japan or China (Fig. 15.5) used for Europe or USA, or from old cultivars or traditional landraces. Then, the adaptation to climatic factors (e.g. winter hardi-

Table 15.5 Sources of resistance and period of time for their integration in commercial cultivars without using molecular markers

Sources of resistance	Period of time (years)
Own breeding populations	Immediately
Adapted cultivars	5–10
Foreign cultivars from similar climate zone	8–12
Non-adapted foreign cultivars	10–15
Landraces, old cultivars, primitive forms	15–20
Wild species, foreign genera	Long term

ness, earliness) and/or to the production system (e.g. plant height, yield level) is often missing. When barley mild mosaic virus (BaMMV) first occurred in Germany in the 1970s, resistances were only found in an old Dalmatian landrace and in Japanese cultivars. Similarly, the re-occurrence of *Fusarium* head blight (FHB) epidemics in the Midwestern USA and Canada during the 1990s promoted the use of Chinese landraces for FHB resistance. Today, the use of molecular markers makes introgression from non-adapted sources more rapid. The use of wild species of the same crop or even from different genera is normally restricted to scientific institutes and universities, but had some impact in the past. Several new resistance genes for barley/powdery mildew (*Blumeria graminis*) were extracted from Israeli wild barley (*Hordeum vulgare* ssp. *spontaneum*) (Jahoor and Fischbeck 1987) and are now used in European barley breeding (e.g. R genes *Mlf*, *Mlt*). Several rust resistance genes in hexaploid wheat (see Table 15.8) are from wild progenitors (*Triticum dicoccoides*, *T. tauschii*), the secondary (*Aegilops*, *Secale*) or even tertiary gene pool (*Leymus*), and in recent sunflower and potato cultivars many resistances have been used from wild species introgressed by sexual crossings, often followed by embryo culture. For example, late blight resistance in potato was introduced from wild species *Solanum nigrum* and *S. villosum* (Colon et al. 1993). However, resistances from distantly related hosts are not more durable than resistances from closely related material (examples in Table 15.8).

Today, also genes from non-hosts or even other kingdoms (viruses, bacteria) inducing disease resistance can be transferred via gene technology (see Sect. 15.7).

15.4 Classical Breeding Strategies

The main problem to be solved in practical breeding programs is the combination of disease resistances with a multitude of agronomic and quality traits. Basically, three breeding strategies are possible that depend on the availability of resistance sources and the type of resistance (Table 15.6). All methods can be used in self- and cross-pollinated crops.

Breeders tend to use resistance sources from the adapted gene pool at first because they are fearful of introgressing genome segments with negatively-acting loci from foreign materials, thus applying recurrent selection or multi-stage selection. Using

Table 15.6 Breeding strategies for improving disease resistance

Strategy	Aim
Backcross breeding	Qualitative resistances from foreign, non-adapted material or wild species
Recurrent selection	Quantitative resistances from own breeding populations/adapted cultivars with a low initial resistance level
Multi-stage selection	Qualitative or quantitative resistances from adapted sources that can directly be combined with agronomic and quality traits

exotic resistance sources via backcross breeding, the agronomic performance of progenies might drop drastically in the first BC generations. This is mainly true, when crossing with sources of quantitative resistances that are based on several genes.

15.4.1 Backcross Breeding

Backcross (BC) breeding aims for the introgression of a target gene from a donor into the genetic background of a recipient genotype used as recurrent parent. This is the classical method for introgressing individual R genes from foreign sources into elite breeding material aimed at clone, line or hybrid cultivars (Fig. 15.6).

With each backcrossing step, the recurrent parent genome enriches. Starting with BC_1, after each backcrossing a selection for the desired resistant phenotype (Aa) is necessary. When aiming for line cultivars or inbred lines, the heterozygous plants should be selfed after the last BC step and selected again to produce homozygous progeny (AA) in the recurrent parent background. At the end, near-isogenic lines are produced that mainly differ in the resistance gene. In practical breeding, often the recurrent parent is changed from generation to generation to keep up with the general selection gain. The number of backcross generations needed depends on the genetic difference between donor and recurrent parent. The larger the gap of performance between both, the more backcross generations are necessary to end up in an agronomically reasonable near-isogenic line. Backcrossing of recessive genes takes more time, because after each BC generation a selfing step has to be performed to produce resistant, homozygous (aa) progeny for selection.

15.4.2 Recurrent Selection

Recurrent selection (RS) was designed to increase the frequency of desired alleles for quantitatively inherited traits by repeated cycles of selection and recombination and simultaneously maintaining genetic diversity (Hallauer and Carena 2009). RS schemes must be designed according to the reproductive system of the crop. In cross-pollinated crops, normally test crosses are produced to analyze the phenotype

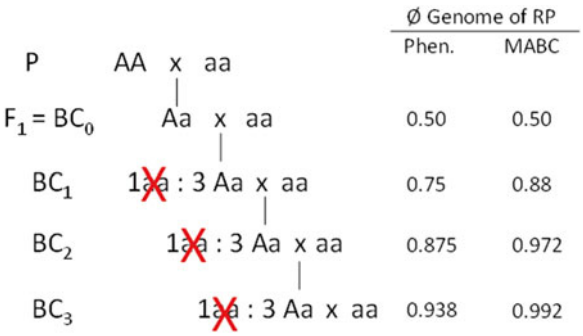

Fig. 15.6 Principle of backcrossing (BC) a single, dominant resistance gene (AA) with a recurrent parent (RP, aa); the average genome proportion of RP is given for phenotypic and marker-assisted backcrossing (MABC, see Sect. 15.5.2; after Openshaw et al. 1994). After each BC susceptible genotype aa must be discarded by resistance tests or marker selection

of experimental hybrids and to select for dominant resistance genes. In self-pollinated crops additional selfing steps are necessary to increase the effect of the desired, mostly additively inherited genes. The main advantages besides increasing allele frequencies and, thus, resistance level of the population are (1) the possibility to test in several locations and/or years in early generations, (2) to simultaneously improve disease resistances and other agronomic and quality traits and (3) the direct use of selected progenies in breeding commercial cultivars.

While RS in cross-pollinating crops like maize is a standard procedure (Hallauer and Carena 2009), Parlevliet and van Ommeren (1988) demonstrated the selection progress by RS in a self-pollinating crop. Their unselected source population had a similar susceptibility to barley leaf rust like a fairly susceptible cultivar. After two selection cycles and several selections within one cycle disease severity was reduced to less than 10 %. The best line had a considerably higher resistance than the most quantitatively resistant commercial cultivar. In the wheat/FHB pathosystem, the realized selection progress from RS after two cycles of phenotypic selection for disease severity were 3.2 % and 2.1 % per year in spring and winter wheat, respectively (Miedaner et al. 2008a; Wilde et al. 2007). A similar selection progress in this pathosystem was reported in China (Jiang et al. 1994).

A challenging task for the breeder with RS is when agronomic traits are negatively associated with quantitative resistance. In many pathosystems with soil-borne fungi, resistant progenies are later in development and/or taller (Fig. 15.7), (Parlevliet 1989; Voss et al. 2008) and this association is even stronger by using natural infections. For practical reasons, however, the farmers in intensive agricultural systems prefer early and short genotypes. This can be handled only by substantially increasing population size and a reduced selection intensity for resistance, earliness and shortness than usual.

Fig. 15.7 Two winter wheat genotypes differing in quantitative resistance to septoria tritici blotch. Note the lateness and tallness of the more resistant genotype on the *left*

15.4.3 Multi-stage Selection

In modern plant improvement programs, selection is a continuous process, and several successive resistance screenings may be applied in a single generation. Different combinations of traits are selected in successive generations depending on the heritability, degree of dominance and seed availability (Cunningham 1975). Selection intensity at each stage is adjusted to the inheritance of traits and exactness of assessment. Figure 15.8 shows selection steps for resistance traits in a modern breeding scheme for line cultivars using doubled-haploids (DH). DH lines have been adopted by barley and maize breeders worldwide and are under development in wheat breeding. They are produced either by in vivo parthenogenesis (maize, wheat) or by androgenesis (barley) and involve tissue-culture techniques (embryo rescue or plating of anthers/microspore, respectively). This procedure allows achieving fully homozygous lines after chromosome doubling in one step. The main advantages are saving time, getting a hand on the final product in early breeding stages, higher selection intensity and accuracy, especially for quantitatively inherited traits. The main disadvantages are higher costs in some crops and only one round of recombination.

During DH0 generation, single-plant tests in the seedling stage or marker-assisted selection (MAS) for single genes (*Pch1*) or QTL (e.g. Fhb1-5) are possible. Highly heritable traits, like qualitative rust resistances, plant height, flowering date etc. can be selected in DH1 single rows on several locations. Quantitative resistances with lower heritability are selected in DH2 and DH3 generations together with grain yield, when larger plots and more environments are available. An advantage of

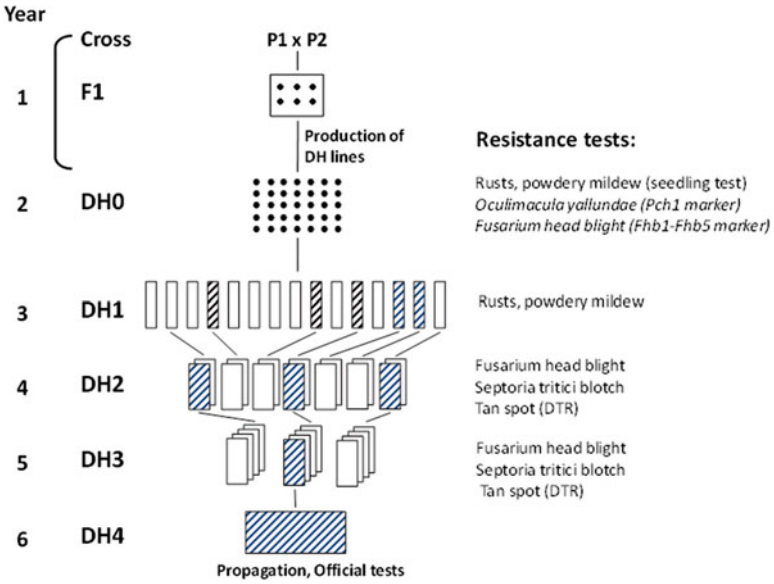

Fig. 15.8 Breeding scheme for self-pollinating crops using doubled haploid lines (DH) and possible selection steps for disease resistances in wheat

multi-stage selection is the probability of finding rare recombinants uniting multiple resistances and superior agronomic traits.

Genetic gain from selection for quantitative traits can be improved by increasing (1) selection intensity, i.e. the fraction of selected progenies relative to tested ones, (2) genetic variation and (3) exactness of disease assessment (heritability) Allard (1960). However, increasing selection intensity means larger populations to select from; increasing genetic variation must include the positive fraction of the desired traits, and increasing exactness means larger plots, more locations and/or more years for testing.

15.5 Marker-Assisted Breeding Strategies

Molecular markers have several advantages. They can be assessed in high-throughput technologies at a very early growth stage with high heritability and they are now relatively cheap. Markers are especially valuable for adult-plant resistances or traits with low heritability, traits that are difficult, expensive and time consuming to assess phenotypically, recessive traits or those controlled by multiple genes (Koebner 2003; Young and Tanksley 1989). MAS offers the advantage of fewer breeding cycles and compilation of several desired traits in one genotype.

The main questions to be solved are the identification of genes/QTLs with high effects, the availability of closely-linked markers and the independence of marker

detection from genetic background (*diagnostic marker*). Ideally, the marker is based on the sequence of the gene of interest (*perfect marker*), but today this is still the exception rather than the rule in agronomically-important crops. Technically, several possibilities for marker analyses are available. For single-marker assays, the competitive allele specific PCR (KASPar) assay has quite recently emerged (Chen et al. 2010). KASPar is a SNP detection system, which is cost-effective for genotyping small subsets of SNP markers. For high-throughput screening, whole-genome array based assays, like the diversity array technology (DArT) (Jaccoud et al. 2001) or the Infinium HD assays (http://www.illumina.com/technology/infinium_hd_assay.ilmn), e.g. for wheat and barley, have been developed. Because both techniques are based on the same marker technique, they can be combined when a SNP set has been established that is inter-convertible between both assays. Older marker techniques, like the single-sequence repeat marker, are still widely used but more expensive per data point and less versatile.

15.5.1 Monogenic Traits vs. Quantitative Trait Loci

For monogenic traits, modern marker detection is straightforward. Based on rather small segregating populations, either F_2 derived, recombinant inbred lines (RIL) or DH populations, a low density SNP assay will suffice to chromosomally localize the underlying resistance gene when phenotypic effects are estimated in the lab for all-plant resistances or on a few field locations. Subsequently, the identified genome segment can be enriched by additional SNP markers. Most closely linked SNPs should be analyzed for their independence from the genetic background of the mapping population and can afterwards be used in breeding populations.

A QTL is a section of a chromosome that affects a phenotypic trait to some extent (Alonso-Blanco et al. 2006). For QTL detection, each individual of a segregating progeny is genotyped for DNA markers and phenotyped for quantitative resistance. The resulting data sets are analyzed biometrically to identify significant associations between marker and traits (St. Clair 2010). QTL mapping is more resource demanding than detection of monogenic traits, because population size should be higher, several locations and/or years are necessary for phenotypic analyses and markers across the whole genome are needed. Piepho (2000) showed in a theoretical study that the power of QTL detection does not considerably increase if the distance between adjacent polymorphic markers is smaller than 10 cM. This indicates that population size is a limiting factor for QTL detection rather than marker density. Currently, two basic techniques are available: bi-parental mapping and association mapping. While biparental mapping employs structured segregating populations with only a few recombinations, association mapping uses a large array of genetically unrelated entries and historical recombination events (Zhu et al. 2008).

Numerous studies, however, have shown that many quantitative resistances are conditioned by a great number of QTLs with small effects. Re-analyzing 85 QTL

studies of 18 crops, Kover and Caicedo (2001) found an average of 4.6 QTLs per pathosystem with a range of 0–18. An individual QTL explained on average about 20 % of phenotypic variance and the range was 0–87 %, where the high values may refer to monogenic resistances with partial effects. Rosewarne et al. (2013) summarized more than 140 QTLs assigned to 49 chromosomal locations for stripe rust resistance in wheat, published during the last 10 years. Many QTLs have been found for leaf rust resistances as well (see: Buerstmayr et al. 2014). Likewise, for FHB resistance, more than 170 QTLs were reported, but they could be assigned to 19 meta-QTL (Löffler et al. 2009) illustrating that most QTLs have been detected more than once in different populations. Other aspects of genetic architecture revealed by QTL mapping include genotype-by-environment interaction, QTL x QTL interaction (epistasis), and pleiotropy, i.e. the effect of one QTL on more than one trait (Young 1996).

15.5.2 Marker-Assisted Backcross Breeding (MABC)

Markers are an ideal tool for accelerating the timely backcross (BC) procedure. Backcrossing with monogenically inherited traits is simple and fast. Objectives are (1) tagging the gene of interest (foreground selection), (2) selecting individuals that are homozygous for a maximum of recurrent parent alleles in a given BC generation (background selection) and (3) reducing linkage drag. MABC is of special advantage when recessive alleles should be backcrossed and the target gene is expressed at a later stage in plant development (adult-plant resistance). While backcross breeding with phenotypic selection is mainly restricted to monogenic resistances, MABC can also be used for introgression of several genes/QTLs.

For BC, the aim is to introduce the target gene into the elite background and to recover a maximum percentage of recurrent parent genome as early as possible with a minimum of marker costs. The BC procedure was optimized for one or two dominant genes (Frisch and Melchinger 2001a; Frisch et al. 1999) or a recessive gene (Frisch and Melchinger 2001b) by simulation studies with respect to population size, marker data points, number of backcross generations, selection strategy and marker systems. As an outcome, a multi-stage selection approach with increasing population sizes from generation BC1 to BC3 considerably reduces the number of required marker data points and, thus, program costs.

Background selection by molecular markers was proposed by Tanksley et al. (1989) and found to accelerate recovery of the recurrent parent genome by two or three generations (Fig. 15.6), Openshaw et al. (1994). While a recurrent parent genome proportion of 99.2 % can be reached by MABC as early as in the BC3 generation, conventional BC has to be prolonged until BC6 generation to gain the same effort. The cost-effectiveness of background selection for gene introgression can be increased with a two-step procedure (Herzog and Frisch 2011): (1) in early backcross generations, when a high number of marker data points is needed, high-throughput assays are advantageous (2) in advanced backcross generations when only those marker loci should be considered that are not yet fixed for the desired alleles single-marker assays are more effective.

During BC, the donor chromosome segment around the target gene can remain fairly long over a large number of backcross generations (linkage drag). For example, Young and Tanksley (1989) found lengths up to 51 cM of the segment attached to a resistance gene after six backcross generations in tomato. Their experiments confirmed theoretical results of Stam and Zeven (1981) who showed that the length of the donor chromosome segment attached to a target gene on a 100-cM chromosome after six backcross generations without background selection is expected to be 32 cM. Moreover, there are numerous examples of undesirable traits tightly linked to a target gene, which were introgressed together with the gene of interest (Zeven et al. 1983). This is especially a risk when the donor is not adapted and fairly different from the elite recurrent parent in agronomic performance. For reducing or even avoiding linkage drag, the sequential analysis of several markers surrounding the target gene has been proposed by Tanksley et al. (1989). First, a fairly distant flanking marker should be analyzed to search for a single or double recombinant. Subsequent analysis of more tightly linked markers can be used to find the individual with the shortest intact chromosome segment (Frisch and Melchinger 2001c).

In conclusion, often disease resistances must be introduced from foreign sources (Fig. 15.9). When combining the phenotyping and genotyping process the respective genes/QTLs can be used directly for introgression into breeding populations by markers. Depending on the relatedness of the source, combination with agronomic traits might be indispensable. Using markers, however, will shorten the process until an improved cultivar is available.

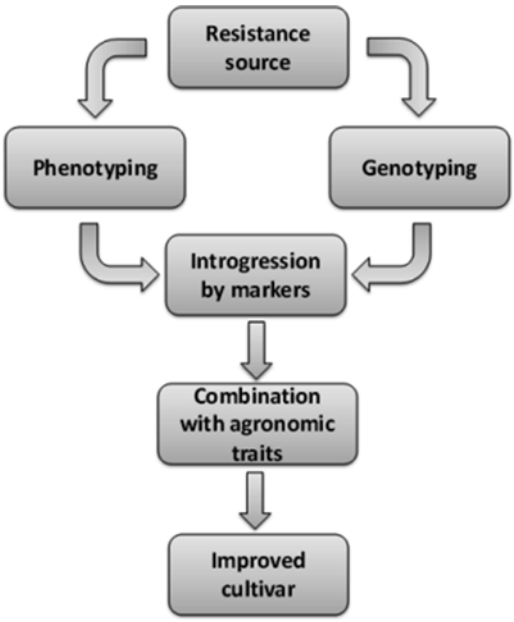

Fig. 15.9 Integration of classical (phenotypic) and marker-assisted strategies to accelerate the introgression process

15.5.3 Pyramiding Resistance Genes and Stacking of QTLs

The strategy of gene pyramiding aims to accumulate several R genes that have been identified in multiple parents into a single genotype that is homozygous for all target loci (Joshi and Nayak 2010). The main aims are a prospected higher durability when several resistance genes act simultaneously in one cultivar against the same disease or achieving a resistance directed towards many races. Fast progress is possible to any combination of genes irrespective of their phenotype using molecular markers. Markers are the only possibility to combine several R genes that are still effective, because their phenotypes cannot be distinguished. Pyramiding genes/QTLs involves two steps: (1) assembling all target genes in a single genotype by multiple crossings and (2) fixation of the target genes in a single, homozygous genotype (Fig. 15.10). The easiest way to combine multiple genes is by a symmetrical crossing scheme involving several single and double crosses and selection of the target genes in a heterozygous state (Peng et al. 2014). For fixation of the genes, a F_2-enrichment strategy (Bonnett et al. 2005) is proposed to counter the demand for large population sizes due to the extreme low frequencies of the desired genotype. For example, the estimated frequency of individuals with eight genes in a homozygous state in one generation equals $(0.25)^8 = 0.00001526$ (=0.001526 %). Using F_2-enrichment, in the first selfing generation genotypes with all target genes either in homo- or heterozygous state are selected. In a second selfing generation, those genotypes with all genes in a homozygous state are selected. Then, probabilities for

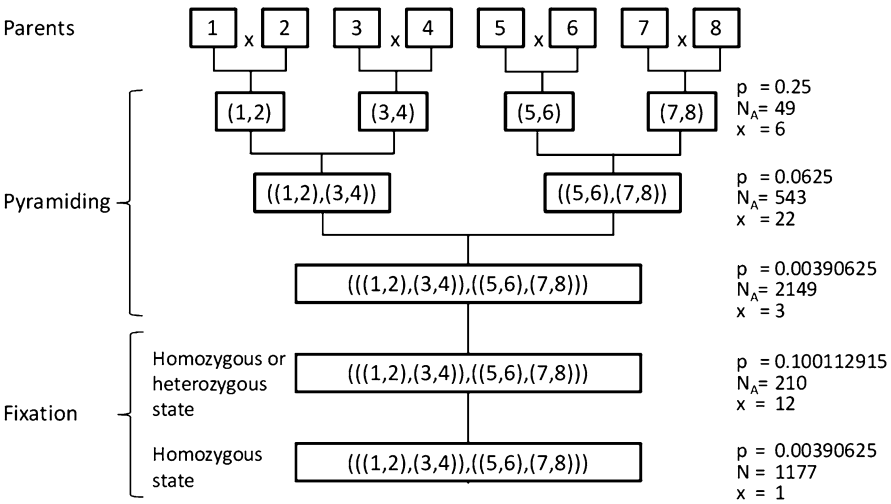

Fig. 15.10 Pyramiding eight genes (1–8) in a single genotype with the frequencies of the desired genotype (p), required population size adjusted for seed needs in the next generation (N_A), number of selected individuals (x) assuming a 99 % success rate and a complete linkage between marker and target gene (Source: After Peng et al. 2014)

seldom occurring recombinants are much higher (Fig. 15.10). This procedure is also used for combining several transgenes in one genotype, e.g. several *Bacillus thuringiensis* (*Bt*)-derived toxin genes (Gahan et al. 2005).

In all pyramiding projects it should be assured that the target genes are inherited independently and provide different resistance mechanisms or avirulence patterns. Assembled Bt genes, e.g. are immunologically distinct and have different binding targets (Gahan et al. 2005). Another example is the pyramiding of three resistance genes for barley yellow mosaic complex, *rym4*, *rym9*, *rym11* (Werner et al. 2005). Two of the genes have already been cloned (*rym4*, *rym11*) and provide different resistance mechanisms. Pyramiding became necessary because virus strains virulent to the *rym4* locus arise in Europe and *rym11* confers broad-spectrum resistance to all known European strains to date (Habekuß et al. 2008). Pyramiding strategies are extremely useful in perennial crops due to their longevity.

While QTL introgression, stacking is crucial because by definition one QTL provides only a part of the resistance and will not alone reach a sufficient resistance level. In this case, stacking of several resistant QTLs by combining favorable alleles from different parents for the same disease is indispensable. For *Fusarium* head blight resistance, each of three different QTLs have been stacked in spring and winter wheat, respectively, and lines with different combinations of resistance alleles were created to analyze the effect of QTL individually and stacked in spring and winter wheat (Miedaner et al. 2006; Wilde et al. 2008, resp.). In spring wheat, the QTLs Fhb1 and Fhb5 from CM82036 provided strongest effects on disease severity, while the 3A QTL from Frontana had only non-significant effects. Also in winter wheat, two QTLs on chromosomes 2B and 6A gave the greatest reduction in disease severity. Interestingly, in both studies, disease reduction by stacked QTLs was lower than expected from adding the individual QTL effects revealing epistatic interactions with *less-than-additive effects* (den Boer et al. 2014). In winter wheat, resistance alleles were associated with increased plant height. This finding and the fact that in each marker class a significant variation of disease severity was still found illustrates that a phenotypic selection after MAS would be advantageous to create the highest resistance level possible. More examples are available of stacked QTLs in barley, common bean and pepper (Joshi and Nayak 2010; St. Clair 2010). By use of molecular markers it is also possible to combine QTL and qualitative resistance genes with the aim to improve the durability of the latter (see: St. Clair 2010).

15.5.4 Marker-Assisted Selection (MAS)

Marker-assisted selection comprises selection for the presence of the donor allele by a closely linked flanking marker during routine breeding programs. By using DH populations, the genes of interest are immediately fixed, by using segregating populations MAS has to be repeated until a homozygous stage is reached. Today this is a routine procedure for monogenic traits when diagnostic markers are available.

For QTL introgression usually two flanking markers are used delimiting the interval in which the putative QTL was detected (Hospital and Charcosset 1997).

In wheat breeding, about 50 genes have been suggested for MAS, including resistance genes to several fungi, cereal cyst nematode resistance, insects and viruses (Table 15.7).

A similar number of resistance genes is available in barley to leaf rust, powdery mildew, *Rhynchosporium secalis*, *Pyrenophora teres* f. *teres*, barley yellow dwarf virus and barley yellow mosaic virus complex. For most of these genes, robust markers and laboratory protocols are available, but it is not clear from the literature how wide they are really used in practical breeding (Miedaner and Korzun 2012). Popular markers are used for resistance to the barley yellow mosaic virus complex and a few durable resistance genes, like *mlo* in barley or *Pch1* in wheat. Xu and Crouch (2008) concluded in their review that *the use of these markers in selection programs by practical plant breeders has been insufficient and disappointing*. This is especially true for the use of QTL alleles. From the hundreds of QTLs known, only very few are used for MAS, e.g. Fhb1 and Fhb5 for *Fusarium* head blight resistance (Miedaner and Korzun 2012).

Table 15.7 Examples of traits in US wheat breeding for MAS

Disease	Genes/QTLs
Powdery mildew	*Pm34*, *Pm35*
Leaf rust	*Lr19*, *Lr21/Lr40*; *Lr22a*, *Lr25*, *Lr29*, *Lr32*, *Lr39*, *Lr47*, *Lr50*, *Lr51*, *Lr67*, *Lr68*
Stripe/yellow rust	*Yr5*, *Yr15*, *Yr36*, *Yr48*, QTL5A
Stem rust	*Sr2*, *Sr22*, *Sr24-26*, *Sr28*, *Sr33*, *Sr35*, *Sr36*, *Sr39*, *Sr44*, *Sr45*, *Sr47*, *Sr52*
Combined (rust) resistances	*Lr34/Yr18/Sr57/Pm38*, *Lr35/Sr39*
	Lr37/Yr17/Sr38, *Lr46/Yr29/Sr58/Pm39*, *Lr67/Yr46/Sr55/Pm46*
Eyespot	*Pch1*
Septoria tritici blotch	Some *Stb* genes
Fusarium head blight	Fhb1 (QTL), Fhb5 (QTL)
Hessian fly	*H9*, *H13*, *H25*, *H25/H32*, *H31*
Russian aphid	*Dn2*, *Dn4*
Wheat stem sawfly	ss.msub-3BL (QTL)
Aphis	*Gb3*
Wheat streak mosaic virus	*Wsm1*
Barley yellow dwarf virus	*Bdv2*
Soil-borne cereal mosaic virus	*Sbm1*
Nematode resistance	Some *Cre* genes

Source: Compilation after Dubcovsky (2014)

15.5.5 Genomic Selection

Genomic selection (GS) is a further development of marker-based selection (Meuwissen et al. 2001), using not only markers linked to QTLs like in MAS, but all available marker information of the genome to make predictions of breeding values for complex traits (Heffner et al. 2009). Special statistical models have been developed that are capable of estimating a large number of markers. The idea is to search for associations among phenotype and genotype in a large, representative training population that was phenotyped in many environments and genotyped by inexpensive, high-throughput whole-genome marker assays or sequencing technologies. Genomic breeding values (GBV) are estimated from this large data set that is used for selection in other breeding populations that have not been phenotyped. GS could facilitate recurrent selection and improve gain from selection per unit time and cost, especially for quantitative disease resistances with low heritability (Rutkoski et al. 2011). It can also be used for backcrossing quantitative traits from a donor parent. Genomic composition of FHB resistance can be predicted more accurately by using GS models than MAS models. The accuracies of cross-validation were 0.6 and 0.7 for FHB severity in barley and wheat, respectively (Lorenz et al. 2012; Rutkoski et al. 2012). For resistance to septoria tritici blotch (STB) in wheat, GS revealed the doubled effect over MAS; it remained, however, on a low accuracy of 0.32 (Miedaner et al. 2013). Obviously, STB resistance is inherited in a more complex manner than FHB resistance. For selecting quantitative adult-plant resistance to wheat stem rust, GS was found to be superior (Rutkoski et al. 2014). A future opportunity will be to combine marker selection of many, quantitatively inherited traits, including several disease resistances, grain yield and quality traits, by GS simultaneously.

15.6 Durability of Resistance

Johnson (1981) described durable resistance as resistance that remains effective when deployed over extensive hectarage and time in an environment favorable for the disease. Therefore, durability can be confirmed only retrospectively over the course of time.

In the past, many race-specific resistances of wheat and barley to powdery mildew (*Blumeria graminis*) and of wheat to yellow/stripe rust (*Puccinia striiformis*) failed when introgressed alone or in pyramids into commercial cultivars. This was even true for genes like *Yr11*, *Yr13* and *Yr14* that express a partial resistance in adult plants (Johnson 1992). Based on about five decades of experience, resistance to biotrophs which relies on a hypersensitive response and is controlled by a single gene is very unlikely to be durable. It might be worthwhile to select also in these pathosystems for quantitative resistances, a strategy that was successful for powdery mildew in wheat cultivars (Miedaner and Flath 2007), leaf rust in barley

(*Puccinia hordei*) (Parlevliet 1995) and common rust in maize (*P. sorghi*) (Stuthman et al. 2007). In these cases, commercial breeders did not rely on largely ineffective race-specific resistances, but gradually increased the level of quantitative resistance by recurrent selection.

Few race-specific resistance genes have remained effective over a long period (Chen 2007; Lowe et al. 2011; Ren et al. 2012; Yang et al. 2013). Three rust resistance gene complexes have been durable to date: *Lr34/Yr18/Sr57/Pm38* (Lagudah et al. 2009), *Lr46/Yr29/Sr58/Pm39* (Lillemo et al. 2008) and *Sr2/Yr30* (McIntosh 1988; Rajaram et al. 1988; Singh et al. 2011). Several new genes of this type have been detected meanwhile, e.g. *Lr67/Yr46/Sr55/Pm46*, *Lr68*, *Yr54* (Singh et al. 2011). Interestingly, they all confer a non-hypersensitive, partial type of adult-plant resistance. Only a few other, monogenic resistances have been proven to be durable, e.g. *mlo* for barley/powdery mildew (*Blumeria graminis* f. sp. *hordei*), *Pch1* for wheat/eyespot (*Oculimacula yallundae*), *rym4/rym5*, a multi-allelic locus with resistance to barley mild mosaic virus (BaMMV) and barley yellow mosaic virus type 1 (BaYMV-1) (Ordon et al. 2009), *Cf9* for tomato/*Cladosporium fulvum*, *Rx* and *Ry* for potato/virus X and Y. Some of these genes have been cloned (Bueschges et al. 1997; Fu et al. 2009; Krattinger et al. 2009; Stein et al. 2005; Uauy et al. 2005) and perfect markers drawn from the gene sequence are available.

15.6.1 Monitoring Plant Pathogen Populations

Populations of plant pathogens are highly adaptive, especially when R genes are employed leading to directional selection. It is, therefore, important to monitor the respective virulence frequencies in pathogen populations using specific differential sets based on the gene-for-gene interaction (Fig. 15.11). The virulence frequency corresponds to the number of virulent reactions of all tested isolates using a differential line. If the virulence frequency of a naturally occurring pathogen population is low (0–10) or moderate (<50 %), the respective R gene is still effective. Additionally, the most frequently occurring pathotypes of a target region, i.e. the combination of virulences in one individual isolate, should be monitored. This information is necessary to elaborate resistance gene combinations that are still effective. A necessary prerequisite for virulence surveys is a differential set, where each R gene and R gene combination used in a pathosystem should be represented by a homogeneous host genotype (Fig. 15.11). Analyzing a larger number of isolates with a differential set discloses implications about the genetic structure of the pathogen population and the usefulness of resistance genes in this region (see: Chen et al. 2009; Klocke et al. 2013; Miedaner et al. 2012a).

Differential sets are available for all rusts of wheat, common and dwarf bunts and loose smut of wheat, powdery mildew of barley and wheat, Northern corn leaf blight of maize and some other diseases. Plant pathogens can accumulate an astonishing high number of virulences (=virulence complexity). For example, the most complex European wheat yellow rust pathotype to date ('Warrior') is able to affect 13 of the

Diff. line	Gene	Is1 *avr1* *Avr2*	Is2 *avr2* *Avr1*	Is3 *avr1+2*	VF (%)
D1	*R0*	+	+	+	100
D2	*R1*	+	-	+	67
D3	*R2*	-	+	+	67
D4	*R1+2*	-	-	+	33
Complexity		2	2	4	

Fig. 15.11 Model of a set of differential lines (D) and isolates (Is). Compatible (+) and incompatible (−) reactions of three isolates (Is1–Is3) with different alleles of avirulence genes (Avr = avirulent, avr = virulent) inoculated on four differential lines (D1–D4) with no resistance gene (R0), one (R1, R2) or two (R1 + R2) resistance genes. VF refers to virulence frequency of all tested isolates on one differential line (example in *red*), complexity to the number of compatible (virulent) reactions of one pathotype (*blue*) on all differential lines (Source: Flath pers com)

17 R genes deployed (Sørensen et al. 2014). It first appeared in Europe in 2011 and 1 year later it was already the dominating pathotype (GRRC 2014). Similarly, powdery mildew pathotypes occur combining 9 out of 14 virulences (Niewoehner and Leath 1998), and in wheat leaf rust the most complex pathotype was virulent for 13 out of 15 R genes (Mesterházy et al. 2000).

Pathogen populations may change rapidly from year to year. There are numerous examples of resistance genes that lost their effectiveness from 1 year to the next because virulent isolates emerged for the respective genes. This is exemplified by data from a long-term virulence survey for yellow or stripe rust (*Puccinia striiformis*) resistance genes in Germany (Table 15.8). If virulences are present in the rust population, their frequency can be in equilibrium (e.g. *Yr 8*) or may increase rapidly (e.g. *Sp*), mainly dependent on the use of wheat cultivars with the respective R gene and the prevalent pathotypes. Once a resistance gene becomes ineffective, the respective virulence might survive for many years. Virulence for *Yr17*, for example, was firstly detected in Germany in 1997 (Bayles et al. 2000), but the virulence frequency was still high in 2014. The rapid increase of the virulence for the R gene *Sp* is an example of a rapid *breakdown* of the respective resistance by the occurrence of a new pathotype (Warrior) that was able to infect hosts with these gene. Therefore, also the occurrence of the other virulences of this pathotype rapidly increased (e.g. for *Yr6*, *Yr17*, *Yr32*). Some virulences (e.g. *Yr5*, *Yr15*, *Yr24*) are still occurring on a low level, because the respective R genes are not used and/or the virulent isolates might have a fitness penalty. The origin of *Yr* genes from alien sources does not imply a higher durability.

Table 15.8 Development of virulence frequencies (%) for nine selected qualitative resistance genes (Yr) to stripe rust (*Puccinia striiformis*) in Germany

R gene	Virulence frequencies (%)					Origin of resistance
Year of evaluation	2000	2001	2002–2006	2007–2010	2011–2014	
No. of samples	137	92	100	90	230	
Yr6	39	35	45	15	89	*T. aestivum*
Yr17	65	49	29	48	79	*Ae. ventricosa*
Yr32	45	10	19	46	84	*T. aestivum*
Sp	0	1	1	3	51	*T. aestivum*
Yr8	19	20	6	35	19	*T. comosum*
Yr10	0	0	1	30	17	*T. spelta*
Yr24	0	0	0	1	2	*T. tauschii*
Yr5	1	0	0	4	1	*T. spelta*
Yr15	0	0	0	0	1	*T. dicoccoides*

Source: Flath pers com

Note: Warrior pathotype has virulence for R genes *Yr1,2,3,4,6,7,9,17,25,32,Sp*

Ae. Aegilops, *T. Triticum*

To elaborate the population structure and dynamics of necrotrophic plant pathogens, molecular markers are indispensable. Intensive studies have been conducted in the last decade for *Fusarium graminearum* (Gale et al. 2007; Schmale et al. 2006; Talas et al. 2011; Zeller et al. 2004), *Rhynchosporium secalis* (Salamati et al. 2000) and *Zymoseptoria tritici* (Zhan et al. 2003). In all these cases, pathogen populations provided an extremely high genetic variation. For example, for *F. graminearum* 72 % of the total molecular variation available in Germany was found by isolating just 30 infected wheat heads from a single farmers' field (Talas et al. 2011). Even more dramatic, for *Z. tritici* 90 % of the global diversity was detected in a single Swiss wheat field (Zhan et al. 2003). The mentioned pathogens are genetically highly flexible and are able to adjust to changing environmental conditions, but also to fungicides (Talas and McDonald 2014).

15.6.2 Achieving Higher Durability of Qualitative Resistances

The main problem of qualitative resistances is that they assert strong pressure on the pathogen for directional selection. Once an isolate becomes virulent to a R gene by mutation, it may spread on a large scale, making the R gene ineffective. Several suggestions have been made since the 1970s to increase durability either by using a higher diversity within the host or by increasing host complexity (Niks et al. 2011). High cultivar diversity on the individual farm or in the region contributes to a higher durability of resistances, however, this does not suffice to avoid directional selection by the host, because in different cultivars the same resistance gene(s) might be

included and farmers prefer the agronomically-best cultivars. Thus, they are growing a small number of recommended cultivars. In Germany, for example, 156 winter wheat cultivars are on the official list; however, the 10 most grown cultivars are contributing about 50 % to the total multiplication area (Anonymous 2014).

15.6.2.1 Pyramiding Several Resistance Genes

Pyramiding is a popular strategy with the hope that it is unlikely that a sequence of multiple mutations to virulence will occur in the same pathotype. Pyramiding is a primary application area for molecular markers (see Sect. 15.5.3) and has been reported to be successful, e.g. in common bean (Souza et al. 2014), where three effective resistance genes for rust have been combined. However, in many pathosystems this strategy did not provide a durable solution, because (1) the pyramided R genes were often used before individually, so a certain level of virulence was already available in the pathogen populations for each gene and (2) many pathogens can develop virulence combinations by sexual recombination.

15.6.2.2 Mixtures of Cultivars, Line Mixtures or Multiline Cultivars

Mixtures or multilines should provide a higher durability of qualitative resistance because different individual R genes are grown on the same field area. With this concept, the pathogen is confronted with a heterogeneous host that should limit the epidemic by reducing the amount of inoculum in each pathogen generation by three mechanisms (Wolfe 1985): (1) dilution effect: lower density of susceptible plants means a higher distance, (2) barrier effect by resistant plants interrupts spore movement and (3) induction of induced resistance by avirulent spores. These epidemiological effects slow the infection processes of virulent isolates to which the host is normally susceptible. Therefore, mixtures are more effective than just pyramiding R genes in one cultivar. Variety mixtures have to be chosen not only for their different resistance combinations but also for similar agronomical features and end use. They, however, display a heterogeneous phenotype. In multilines, several R genes are crossed by the breeder in different sublines of the same cultivar by recurrent backcrossing. Given a sufficient BC generation the cultivar has a homogeneous phenotype and is easier to handle for the farmer. Cultivar mixtures, however, have the advantage that not only several individual R genes are introduced, but also different quantitative resistances of the mixture partners, if available. Both concepts have shown their effectiveness to wind-borne pathogens in the past (Wolfe 1985; Wolfe and Finckh 1997). In the former East Germany, for example, about 80 % of the spring barley area was grown by three mixtures of four to five cultivars with each cultivar possessing different combinations of R genes to powdery mildew (Frauenstein 1984). All mixtures contained *mlo*, a gene that is still durable despite its wide use. Individual cultivars/R genes from a mixture were changed when the corresponding virulence increased in the population. Besides this *clean crop*

concept, also *dirty crop* multilines including susceptible partners have been proposed (Browning and Frey 1969) that should further reduce selection pressure, thus stabilizing pathogen populations.

Despite their effectiveness in reducing the effect of epidemics, it is a matter of debate whether cultivar mixtures or multilines provide a higher durability of R genes, because often mixtures with only a few components are chosen, R genes in mixtures overlap, and the pathogen might be stimulated to develop more complex pathotypes than by growing cultivars with individual R genes. Moreover, they afford a permanent monitoring of the pathogen populations to determine which R genes are still effective. For private breeders, the multiline concept has never been an option, because it is time and resource demanding; multilines must be developed before the success of a cultivar is known and the lifespan of a cultivar gets shorter in industrial countries. Cultivar mixtures, on the other hand, are often opposed by the purchasing industry (miller, baker, brewer) that insist on varietal identity and purity. They may in addition cause legal problems according to plant breeders' rights because the components of a mixture often belong to different companies, their registration is an additional effort, and they must be deployed at least on a regional, or better on a state basis, to become effective. The latter is also valid for the suggestion to deploy R genes by rotations in time and space. Once an R gene gets ineffective it should be withdrawn before the corresponding virulence achieves a maximum frequency and could possibly be re-used when virulence frequency dropped in the population.

McDonald and Linde (2002) suggested predicting the durability of a resistance by population-genetic parameters of the pathogen, using the genetic diversity of a population (*evolutionary potential*) that is mainly affected by the mode of reproduction (asexual, sexual, mixed), mating system (inbreeding vs. outcrossing), type of migration/gene flow (soil-, water-, airborne) and effective population size. *High-risk pathogens* have a large effective population size, high gene flow by airborne spores over long distances, mixed reproduction system, i.e. sexual outcrossing alternating with asexual propagules, and efficient directional selection by R genes of the host. Examples are powdery mildew (*Blumeria graminis*), potato late blight (*Phytophthora infestans*), wheat stem rust (*Puccinia graminis* f. sp. *tritici*), and crown rust of oats (*P. coronata* f. sp. *avenae*). According to these risk factors they recommended different breeding strategies for using R genes including quantitative resistances for the latter two cases (Table 15.9).

15.6.3 Durability of Quantitative Resistances

For quantitative resistances, not much is known about their durability despite the hope that they might last for a long time. There are some practical examples where resistances have been durable over long periods, but not much is known on the adaptability of pathogen populations to quantitative resistances. This is an inherent

Table 15.9 Resistance breeding strategies related to the evolutionary risk of a pathogen

Strategy	Gene/ genotype diversity	Reproduction/ mating type	Gene/ genotype flow	Risk	Example
Individual R genes	Low	Asexual	Low	Low	*Fusarium oxysporum*
Pyramiding	Low	(Mostly) asexual	High	Moderate	*Magnaporthe grisea*
Regional deployment	High	Asexual	Low	Moderate	*Rhizoctonia solani*
Cultivar mixture	High	Mixed/outcrossing	High	High	*Blumeria graminis*

Source: Compilation after McDonald and Linde (2002)

problem because selection experiments on the pathogen side are time-consuming, changes in pathogen aggressiveness are expected to be small and often the sexual cycle could not be included due to experimental difficulties. For quantitative resistance of winter rye to powdery mildew, no adaptation has been found when five mildew populations were grown asexually on five differently resistant cultivars in the lab across 2 years (Welz et al. 1993). From the molecular point of view, it appears, at the current state of knowledge, that quantitative resistances are composed of groups of functionally diverse genes encoding different pathways.

A quantitatively resistant host cultivar has no directional selection effect on pathogen populations, because no specific genotype-by-isolate interaction occurs. However, growing hosts with a high level of quantitative resistance on large hectarage might lead to an increased aggressiveness level of nonspecifically adapted pathogen populations (Voss et al. 2010; Zhan et al. 2003). For this evolutionary process, the term *erosion* was proposed (McDonald and Linde 2002). The probability of erosion of quantitative resistances depends on the life style of the pathogen, mainly on the role of the host for survival and the mode and frequency of recombination. For necrotrophic pathogens that can also survive saprophytically, adaptation processes should be slower than for biotrophs because of the episodic nature of selection, i.e. selection to higher aggressiveness is possible only during host passage that might provide different selection factors than the saprophytic phase (Miedaner et al. 2008b). Regular sexual recombination additionally will break positive allelic combinations selected during host passage slowing down erosion of resistances in these pathosystems. Additionally, pathogen populations may vary considerably in their aggressiveness from year to year as a result of genotype-by-environment interactions that occur for both host and pathogen and, thus, affect selection intensity (Voss et al. 2010). Nevertheless, a gradual adaptation of the necrotrophic *Cochliobolus heterostrophus* causing southern leaf blight of maize was detected after three generations of selection on a quantitatively resistant inbred line (Kolmer and Leonard 1986). Leaf rust populations showed a quantitative increase in the area under a disease progress curve when grown on two out of three quantitatively resis-

tant wheat genotypes across five selection cycles (Lehmann and Shaner 1996). The authors concluded from their experiment that *quantitative resistance may inherently be more durable than hypersensitive resistance, but it must nonetheless be managed intelligently if it is to provide durable resistance*. This might be valid for all pathosystems with quantitative resistances.

15.7 Transgenic Approaches

Genetically modified (GM) crops have been commercially grown since 1996. From the beginning, insect resistance mediated by the toxins of the soil bacterium *Bacillus thuringiensis* (*Bt*) were used. The first targeted insect was corn borer (*Ostrinia nubilalis*), soon followed by corn root worm (*Diabrotica* spp.) (Dunwell 2014). At the end of 2013, an estimated 28.8 million hectares of land were planted with crops containing *Bt* genes worldwide (ISAAA 2014). Past or present commercialized Bt crops and their respective *Bt* genes include cotton (*cry1Ac*, *cry2Ab2*, *cry1Fa2*), maize (*cry1Ab*, *cry1Ac*, *cry1Fa2*, *cry3Bb1*, *cry9C*), and potato (*cry3Aa*) (Shelton et al. 2002).

In the US, 93 % of all major crops are GM cultivars, 76 % of them have two or more genes for Bt and herbicide resistances stacked together. From the beginning, a refuge strategy was used, i.e. areas of non GM plants that should delay pest resistance to Bt crops. Refugia have been mandated e.g. in the United States and Australia. The concept underlying the refuge strategy is that insect resistance to Bt toxin is recessively inherited. Pests surviving on Bt crops will mate with the relatively abundant susceptible pests from nearby refuges of host plants without Bt toxins (Tabashnik et al. 2013) thus reducing the incidence of insects carrying a mutant gene homozygously. Recently, a review on the lessons *from the first billion acres* clearly showed that insect resistance to Bt can be delayed but not prohibited (Tabashnik et al. 2013). Most insect populations remained susceptible to Bt toxin but reduced efficacy of Bt crops has been found for some populations of 5 out of 13 analyzed pests in comparison to only one pest species with resistant populations in 2005. A main reason for the development of resistant insects is that the refugia strategy was not adopted by all farmers or refuge areas were too small. New approaches to enhance durability of *Bt* genes are the combination of *Bt* genes with different modes of action in one cultivar or the distribution of seed mixes with Bt and non-Bt seeds (Dunwell 2014). Future developments will be engineered chimeric Bt toxins, binary Bt toxins and hybrid Bt toxins targeting multiple insect orders (Huesing and English 2004). Additional crops (e.g. apple, broccoli, cabbage, tobacco, tomato, soybean, rice) have also been engineered to express *Bt* genes. A beneficial side effect of Bt resistance is the lower susceptibility to *Fusarium* ear rot in maize, because the fungus uses the insect damage as the portal of entry (Munkvold et al. 1997).

In conclusion, GM crops have the same problems with durability of resistance genes than cultivars with monogenic, qualitative resistances, and in fact, in an evolutionary context there is no difference.

No commercial GM crops for fungal resistance are available to date although a lot of research has been invested (Dunwell 2014). Among the fungal diseases in cereals, *Fusarium* head blight is one of the most important. Many plant defense genes have been reported to confer a quantitative resistance to this pathogen or reducing mycotoxin contents (Becher et al. 2013). Typically, reductions of 20–50 % in greenhouse experiments were reported; however, transgenic lines have fallen short of reaching levels of *Fusarium* head blight resistance comparable to those of highly resistant cultivars.

Newer concepts are the use of native resistance genes and transferring them to other cultivars or other crops. The company BASF has produced the GM cultivar Fortuna that harbors two broad-spectrum resistance genes from the wild potato (*Solanum bulbocastanum*) against late blight. The GM potato was, however, withdrawn because of the lack of acceptance in the German public. Zeller et al. (2012) pyramided different alleles of the race-specific resistance gene *Pm3* (wheat/powdery mildew) by gene technology and showed that resistance increased with GM richness (0–3 alleles) and GM concentration (0, 50, 100 % of plants containing the transgene). The wheat *Lr34* durable resistance gene to several rusts has been found to provide resistances to several fungal pathogens in barley (Risk et al. 2013) after transformation.

Another concept is the silencing of genes that are indispensable for pathogenicity in the pathogen or of host genes that the pathogen need for infection and multiplication. In maize, silencing of a putative cystatin gene (*CC9*) improved resistance to smut (*Ustilago maydis*) (van der Linde et al. 2012). Silencing the effector gene *Avr10* of the wheat powdery mildew resulted in reduced fungal development in the absence, but not in the presence, of the matching resistance gene *Mla10* (Nowara et al. 2010). The authors speculate on using host-induced gene silencing (HIGS) to control multiple diseases of a given crop because constructs can be designed such as containing multiple stacked RNAi target sequences. Also, multiple transgenic events can be stacked by crossing the corresponding lines.

One the most successful uses of GM crops was the resistance of papaya to papaya ringspot virus by transformation with viral capsid proteins. The first virus-resistant papayas were commercially grown in Hawaii in 1999 (Fereira et al. 2002). Today, they cover three quarters of the total Hawaiian papaya crop.

15.8 Limits to Resistance Selection

Resistance selection has several limitations concerning host and pathogen. Combining resistances against the most important diseases of a crop with superior agronomic features, including grain yield, is still a challenge for the breeder, especially in countries/regions with a high yield level. There, the farmer usually does not tolerate a yield penalty caused by a higher resistance level of a cultivar. It might also be a challenge to combine superior disease resistances with a short plant stature and early maturity of the crop (Fig. 15.12).

Fig. 15.12 View in a commercial winter wheat breeding nursery. Usually, the breeder handles very large numbers of progenies to increase the chance of finding genotypes superior for multiple traits; this affords for resistance selection easy, reliable, and cheap methods for disease assessment, including molecular methods

On the other hand, some pathosystems provide higher progress from selection than others. Causes for a low gain from selection are either restricted genetic variation in adapted breeding populations, or high effort for resistance testing and/or low heritability due to environmental effects. For example, resistances to soil-borne foot rot diseases in wheat and rapeseed usually have a low heritability, because they are highly affected by weather and soil factors. This causes high costs for disease assessment because several stems per plot have to be rated individually. Resistances for newly arising diseases usually show no or low genetic variation in adapted breeding populations. Some pathogen populations change so rapidly that resistance selection seems to be a lost race. Moreover, the breeder has to consider a limited budget and thus will concentrate on those resistances having a high economic impact and where no alternative control mechanisms are available. The higher the number of traits to be combined in a cultivar, the lower the selection progress will be because each additional trait reduces the chance of finding a superior genotype when budget and population size are limited. In conclusion, the practical breeder cannot select for all resistances that might be desirable and gain from selection is usually slow because a lot of other traits have to be considered.

15.9 Conclusions and Prospects

Considerable information has been collected on the most popular pathosystems in the last decades and in all *traditional* pathosystems it is quite clear how to select for higher disease resistance; it just has to been done by the breeder consequent upon sufficient selection intensity. Quantitative resistances have been shown to be more durable and should be the long-term breeding goal in any program. They can drive pathogen attack under the damage threshold or at least considerably reduce the yield effect of pathogens during a heavy epidemic.

New techniques of phenotyping let the size of breeding populations grow considerably. This is necessary, because in each crop a combination of resistances is needed and most of them are inherited in a complex basis. In future, global climate change will also affect pathogen and insect populations of a target region like Central Europe in several aspects: (1) arrival of new pathogens with higher temperature requirements, (2) spread of already established pathogens to a larger area, (3) higher multiplication rates and subsequently higher population sizes of temperature-dependent pathogens, (4) decline of cold-adaptive pathogens and (5) changes within pathogen populations to higher adaptiveness to new climatic conditions. For all these points, examples can already be given and it is clear that breeders' work will change but never end.

To compete with future demands, new genetic materials and technologies can support practical breeders. In the last decade, broad-spectrum resistances (BSR) were described that occur not only among R genes (see examples in Table 15.7), but also among QTL-mediated resistance to several pathogen species (Buerstmayr et al. 2014; Miedaner et al. 2012b; Wisser et al. 2005). It was speculated that these BSR QTL are part of the basal defense of the plant (Kou and Wang 2010; Poland et al. 2009) and these loci could open new avenues for achieving durable, combined resistances. Also implementing genomic selection (GS) into practical plant breeding will increase not only the selection gain per year, but also the power to combine complex disease resistances with grain yield and other agronomic traits. Another developing technology is high-throughput sequencing that will allow direct selection of resistance alleles in the genome once more resistance genes from crops are isolated.

Providing new forms of fungal disease resistances by genetic engineering has been an obstacle that is still not overcome. An advantage of transgenic methods is the possibility of introgression of single genes from genetic resources without transferring other genomic segments that potentially have a negative impact. In future, it should be possible to transfer gene cassettes of multiple monogenic resistances into elite breeding material that will be inherited as a single locus. Natural gene cassettes, like the broad-spectrum resistance locus *Lr34/Yr18/Sr57* in wheat could be regulated by synthetic promoters that result in a higher expression level or allow a broader range of pathogens to be controlled. Last but not least, synthetic R genes could be constructed for different pathogen ranges and expression levels that could be modified according to the evolution of pathogen populations.

The major challenge for the breeders in future, however, is the same as in the past: to keep up with the evolution of pathogens by selection for host resistances. With the new enabling techniques, the breeder might get an advantage for the first time.

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Chapter 16
Breeding and Genetics of Resistance to Fusarium Wilt in Melon

Ali Oumouloud and José M. Álvarez

Abstract Melon Fusarium wilt (MFW), caused by *Fusarium oxysporum* f. sp. *melonis* (Fom), and is an important soil-borne disease of melon worldwide. The four known races 0, 1, 2, and 1,2 of this pathogen can be distinguished by infection on appropriate cultivars. Effective control can be achieved only through host resistance. Two major genes, Fom-1 and Fom-2, control resistance to races 0 and 2, and 0 and 1, respectively; whereas partial polygenic resistance to race 1,2 has been described. Fom-2 gene has been cloned, and the information generated from the LRR region sequences allowed the development of useful functional markers. Also, several molecular markers linked to Fom-1 gene have been reported; nevertheless, their usefulness was variety-dependent. These markers were used for the positional cloning of this gene. More recently this gene was isolated by a map-based cloning strategy. The sequence analysis revealed that Fom-1 belongs to the TIR-NB-LRR type. Resistance to Fom race 1,2 is complex and appears to be under polygenic control. Partial resistance to this race was detected in a few Far Eastern melon accessions, except for the breeding line BIZ where near-complete resistance was described. To date, quantitative trait loci (QTLs) associated with resistance to race 1,2 have been reported only in the line Isabelle and two other breeding lines BIZ and 03MFR001795. This chapter summarizes findings reported in the literature on genetic resources of resistance, molecular markers and quantitative trait loci for resistance to Fom.

Keywords *Fusarium oxysporum* f. sp. *melonis* • Races • Resistance gene • Vegetative compatibility groups • Molecular marker • QTLs

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16.1 Introduction

Melon (*Cucumis melo* L.) is an important world horticultural fruit crop with an overall annual production of 26 million mt and a planted area of about 1.3 million ha (www.fao.org). Fusarium wilt is one of the economically most important diseases of melon. It is caused by *Fusarium oxysporum* f. sp. *melonis* (Fom). This disease was first described in New York in 1930 (Chupp 1930), and later was found in many melon-growing areas worldwide, including elsewhere in the USA (Leach and Currence 1938), Europe and Asia (Quiot et al. 1979; Sherf and Macnab 1986) and Africa (Schreuder et al. 2000). Currently, melon Fusarium wilt (MFW) is widespread throughout most regions of the world where melon is grown.

It is difficult to control this disease through fungicide application and good cultural practices, because the fungus survives in the soil as chlamydospores, and is able of colonize crop residues and roots of most crops grown in rotation with melon (Banihashemi and DeZeeuw 1975). Solarization has been used with good results against soil-borne diseases of melon (Tamietti and Valentino 2006), but it is often limited by local climate constraints such as temperature and relative humidity (Shlevin et al. 2004). Grafting of melons onto resistant rootstocks is also considered a promising practice to control soil-borne diseases in vegetables, particularly for MFW (Cohen et al. 2002; King et al. 2008). However, the added cost still limits its feasibility only to melon varieties with great economic value.

Thus, due to the limited effectiveness of physical, chemical and biological control methods, the use of genetic resistance for MFW control is the best management strategy on a medium- to long-term basis. A better understanding of the mechanisms affecting resistance and of their genetic control as well as the identification of molecular markers linked to resistance genes would enable the pyramiding of different resistance genes. This would be a positive contribution to the development of higher and more durable resistance. The potential benefits of Fom resistance are therefore great because resistant cultivars are the most economical and environmentally acceptable way of controlling the disease.

This chapter summarizes findings reported in the literature on genetic resources of resistance, molecular markers and quantitative trait loci for resistance to Fom.

16.2 Pathogen and Pathogenesis

16.2.1 Species, Races and Vegetative Compatibility Groups

Fusarium oxysporum Schlechtend:Fr. is an asexual fungus that includes both pathogenic and nonpathogenic strains. The latter are defined as strains for which no host plants have been identified. Pathogenic *F. oxysporum* strains can cause disease on more than 100 different plant species. In spite of the broad host range of the species as a whole, individual strains usually infect only a single or a few plant species.

Therefore, phytopathogenic *Fusarium oxysporum* strains have been subdivided into over 100 different host-specific forms (formae speciales or f. sp.) which are morphologically indistinguishable and represent intra-specific groups of strains with similar or identical host range (Baayen 2000; Di Pietro et al. 2003; Lievens et al. 2008; Snyder and Hansen 1940). The formae speciales concept has been useful to plant pathologists because it identifies a subset of isolates that are of concern to the production of a crop susceptible to Fusarium wilt. Nevertheless most studies reported to date have shown that in several cases, *F. oxysporum* f. sp. consist of multiple, independent lineages that evolved polyphyletically through convergent evolution.

Fusarium oxysporum f. sp. *melonis* (Fom) Snyder and Hansen (Leach and Currence 1938), is specific to melon (*Cucumis melo* L.), and it causes a vascular wilt which is considered as one of the most severe disease of melon worldwide. The nature of the diversity comprised within Fom has direct bearing on the prospects for MFW control through genetic resistance; therefore a range of approaches are typically employed for the characterization of Fom strains.

Variation in the virulence (aggressiveness) of Fom isolates has been known for some time (Leach and Currence 1938; Messiaen et al. 1962; Risser 1973; Risser et al. 1969), in 1976, Risser et al. proposed a standard method for the naming of physiological races of this pathogen, and the genes that confer resistance to the disease. Under that system, resistance genes are numbered according to their order of discovery, and races are defined by the corresponding resistance genes in the host that they overcome. Using this system, four races of the pathogen are defined based on the resistance genes Fom-1 and Fom-2 (Fig. 16.1). Race 1 defeats Fom-1 and race 2 overcome Fom-2. The third race, designated race 0, is for isolates that do not defeat any known resistant gene and are, therefore, pathogenic only to universally susceptible cultivars lacking any known resistance. A fourth race, designated race 1,2, is for isolates that overcome both genes and has been further subdivided into race 1,2w, which causes wilting and death without prior yellowing symptoms, and 1,2y, which induces leaf yellowing symptoms before the death of the plant (Fig. 16.2). Race 1,2 is considered as the most virulent affecting melon cultivars to date (Bouhot 1981; Cohen et al. 2002; Luongo et al. 2015). The spread of this race throughout the world has become a problem for melon cultivation (Herman and Perl-Treves 2007; Veloso et al. 2000).

Also, vegetative compatibility groups (VCGs) have been used to group isolates of *Fusarium oxysporum* (Puhalla 1985) including Fom. Vegetative compatibility is controlled in *Fusarium* spp. and other fungi by several vegetative (vic) or heterokaryon (het) incompatibility loci (Leslie 1993). For two individuals to be vegetatively compatible and form a stable heterokaryon, they need to share a common allele at each vic locus (Correll 1991). Currently, nine vegetative compatibility groups (VCGs) have been characterized in Fom worldwide. Jacobson and Gordon (1988, 1990a) identified in the US eight vegetative compatibility groups (VCGs) 0130, 0131, 0132, 0133, 0134, 0135, 0136 and 0137 in a worldwide collection of Fom isolates. Katan et al. (1994) identified two VCG groups, one corresponded to the previously described VCG 0135, and a new 0138 which was further divided into

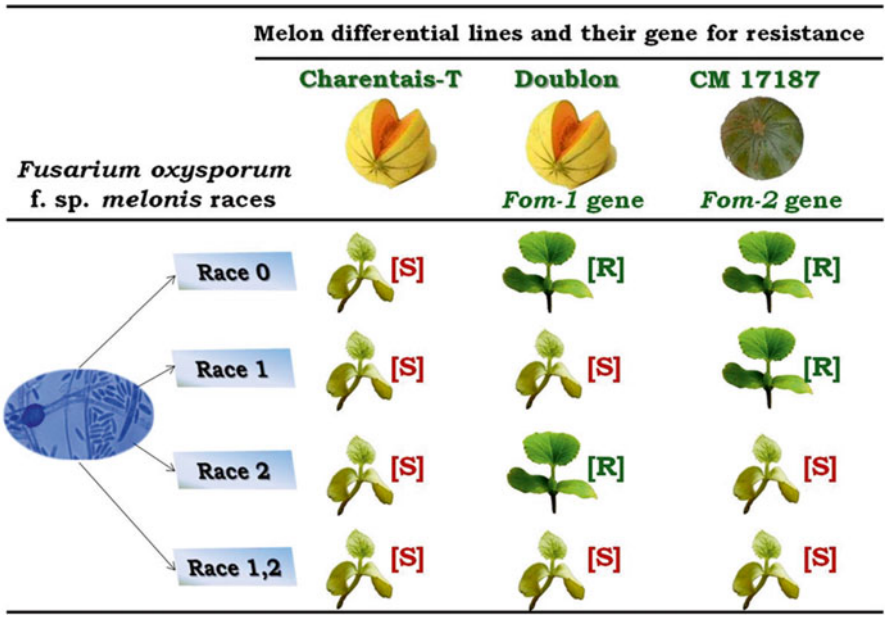

Fig. 16.1 Classification of *Fusarium oxysporum* f. sp. *melonis* races based on Risser et al. (1976). *S* Susceptible, *R* Resistance (Source: Oumouloud 2008)

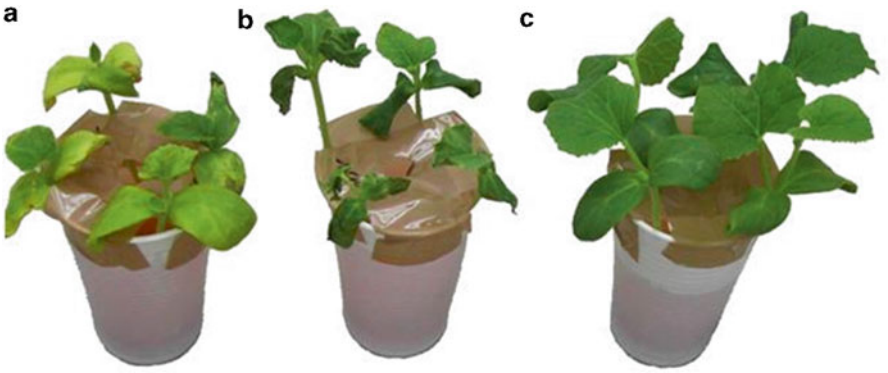

Fig. 16.2 Typical symptoms caused by *Fusarium oxysporum* f. sp. *melonis* race 1,2 in melon under artificial inoculation: (**a**) Yellowing of susceptible plants induced by 1,2y pathotype. (**b**) Sudden wilt of susceptible plants induced by 1,2w pathotype. (**c**) Symptomless plants identified as resistant to both pathotypes (Source: Oumouloud 2008)

two subgroups. Also three undesignated VCGs were identified among 13 isolates in Italy (Gennari and D'Ercole 1994) and 72 tested isolates from South Africa were found to belong to VCG 0134 (Schreuder et al. 2000). Additionally, among 19 tested isolates from Greece, seven belonged to VCG 0138, and the others were associated with an unclassified VCG (Elena and Pappas 2006).

Furthermore, the genetic diversity among isolates within Fom isolates has been examined by using various genome-wide techniques, such as mitochondrial DNA (mtDNA) (Jacobson and Gordon 1990b), nuclear ribosomal DNA intergenic spacer (IGS) region (Appel and Gordon 1995), DNA fingerprinting (Schroeder and Gordon 1993), repetitive extragenic palindromic PCR (Rep-PCR) (Mirtalebi et al. 2013) and random amplified polymorphic DNA (RAPD) (Luongo et al. 2015). Moreover, genes containing conserved sequences, including at least one intron such as translation elongation factor (TEF-1α), have also been used to characterize the variability occurring within and among Fom races (Luongo et al. 2015).

Most of these studies revealed a complex relationship between VCG and races in Fom. Isolates of all four races of Fom have each been found in more than one VCG, indicating that no race represents a genetically homogeneous group of isolates, and, in contrast, all four races can be present in a single VCG. Nevertheless, Fom races 0, 1, and 1,2 are all associated with identical mtDNA (Jacobson and Gordon 1990b) and nuclear DNA haplotypes (Schroeder and Gordon 1993). Furthermore, using both RAPD and TEF-1α analyses, Luongo et al. (2015) showed the close relationship between the three Fom races 0, 1 and 1,2 on one side and the phylogenetic distance of race 2 on the other.

The race groupings in Fom conceal known diversity in symptomatology, vegetative compatibility and molecular techniques such as mtDNA, IGS region, DNA fingerprinting, RAPD markers, Rep-PCR and sequence comparison of TEF-1a gene polymorphism. Furthermore, the current practice of categorizing races may not be adequate in ascertaining the entire virulence diversity within Fom. As mentioned above, Fom races are recognized by interaction with melon resistant lines, so that with two resistant genes Fom-1 and Fom-2, the four known races in Fom (0, 1, 2 and 1,2) represent the maximum that can be described by the present system. If additional melon resistance genes were available, more races might be identified, possibly changing our perception that different VCGs are identical in virulence.

Finally, it is currently impossible to characterize the genetic basis for the virulence phenotypes in Fom, thus it is difficult to establish the weight that should be accorded to this character. Traits, other than virulence, that can be used to group strains within Fom also provide only partial measures of the underlying diversity. The correlation between molecular techniques and VCG helped confirm the importance of VCG as indicator of genetically isolated population within Fom. However, the race diversity within a VCG should not be ignored. Studies using molecular approaches also have shown additional genetic polymorphism within VCGs. Thus, there probably is no one trait, physiological, genetic or molecular, that alone will provide an entirely satisfactory basis for Fom isolates classification.

16.2.2 Origin of Races

The appearance of a new race in a geographic location indicates either a recent introduction from areas where the race was already established (Gordon and Martyn 1997; Jimenez-Gasco et al. 2004) or an independent evolution from an existing race

(Cai et al. 2003). The literature offers some evidence to support each explanation for the origin of new pathogenic races.

Although Fom isolates comprise considerable diversity and are associated with multiple VCGs, it is often possible to show a close relationship between isolates responsible for infestations in multiple locations. For example, the race1/VCG 0134 is known to occur in Europe (Jacobson and Gordon 1990a), Central Asia (Mohammadi et al. 2004), North America (Jacobson and Gordon 1990a) and South Africa (Schreuder et al. 2000). Molecular markers, including mitochondrial (Jacobson and Gordon 1990b) and nuclear DNA haplotypes (Appel and Gordon 1995), confirm that VCG 0134 corresponds to a clonal lineage. These results provide evidence of the widespread occurrence of individual genotypes.

On the other hand, some results reported in the literature support the view that simple genetic changes can generate altered pathotypes. The best evidence of how this process could occur comes from the polyphyletic of Fom. In some cases, the close relationship between pathogenic races implied by their association with the same VCG has been corroborated by other measures of genetic relationship. For example, VCG 0134 of Fom is associated with all four known races in this forma specialist. Furthermore, race 0, race 1, and race 1,2 all are associated with identical mtDNA (Jacobson and Gordon 1990b) and nuclear DNA haplotypes (Schroeder and Gordon 1993). The close relationship between three different races may indicate that relatively simple genetic changes can lead to a change in cultivar specificity, i.e. one pathogenic race can give rise to another.

Furthermore the existence of both pathogenic and non-pathogenic strains in *Fusarium oxysporum* indicates that the derivation of one from the other must be considered possible as strongly supported by the most recent horizontal gene transfer evidences (Ma et al. 2010; Rep and Kistler 2010).

16.2.3 Genome Structure

More recently, the genome of Fom strain NRRL 26406 have been sequenced (Ma et al. 2014) and their size was estimated to be 68 Mb. This genome contains a total of 61 rRNA, 311 tRNA, and 20,033 protein-coding genes. An insightful comparative study using one *Fusarium oxysporum* strain isolated from a diseased tomato plant revealed structural and functional compartmentalization of the genome (Ma et al. 2010). This finding was further confirmed by optical mapping and genomic sequencing of all 10 *F. oxysporum* strains examined to date, including the Fom strain NRRL 26406 (Ma et al. 2014).

It was established that the *Fusarium oxysporum* genome can be structurally divided into the *core* and the *accessory* genomic regions (Ma 2014). The core genomic regions, which reside on conserved chromosomes, are preserved among all *Fusarium* species and are present in all *F. oxysporum* strains, regardless of their hosts. These core chromosomes contain all housekeeping genes and few transpos-

able elements (Ma 2014). The conservation of the core indicates vertical transmission.

In contrast, the accessory genomic regions, in the form of transposon-rich pathogenicity chromosomes, also called lineage-specific (LS) chromosomes and regions are absent from other sequenced *Fusarium* genomes, except those that share the same host. The genes encoded in LS regions differ in their phylogenetic history from the genes on the core chromosomes (Ma et al. 2010; Rep and Kistler 2010). These pathogenicity chromosomes are horizontally transferred and contribute directly to the host specificity. In fact, by comparative genomics Ma et al. (2010) showed that *Fusarium oxysporum* f. sp. *lycopersici* contains lineage-specific (LS) chromosomes enriched for genes related to host-pathogen interaction and demonstrated the transfer of these chromosomes between strains of *F. oxysporum* converting a non-pathogenic into a pathogenic strain.

16.2.4 Epidemiology and Defense Responses

Dissecting the infection strategies and understanding the molecular pathways involved in Fom pathogenesis have been and continue to be the subject of intensive research. Such knowledge will not only increase our overall understanding of the disease process but also will contribute to the development of strategies for melon Fusarium wilt management in agricultural settings.

In the absence of roots, Fom survives in the soil either as dormant propagules (chlamydospores) or by growing saprophytically on organic matter (Gordon et al. 1989). The proximity of melon roots induces the dormant propagules to germinate and initiate infection. In general, plant infection by the Fom process comprises several stages of host-pathogen interaction: recognition of the host roots and adsorption; penetration of hyphae through the different root tissues; penetration and progression in the xylem and adaptation to the internal plant environment. During the final stage of infection, the fungus secretes lytic enzymes and toxins that lead to disease symptoms, including necrotic lesions, chlorosis and wilting.

Over the last two decades, 29 pathogenicity-related genes of *Fusarium oxysporum* have been detected (Michielse and Rep 2009). At least four pathogenicity-related genes of Fom involved in root penetration and colonization have been discovered to date: (1) Arg1 gene, which encodes argininosuccinate lyase essential for the Arg biosynthesis and virulence of Fom (Namiki et al. 2001), (2) the mitochondrial protein gene Fow1 that has been identified as a virulence determinant of plant pathogenic fungi (Inoue et al. 2002); (3) Fow2 gene, a Zn(II)2Cys6-type transcription regulator, that controls the expression of genes involved in the pathogenic program of Fom and (4) snt2 gene, a transcription factor encoding gene involved in the pathogenicity, hyphal growth and conidiation in Fom (Denisov et al. 2011).

Studies using molecular techniques such as green fluorescent protein (GFP) marker (Imazaki et al. 2007; Inoue et al. 2002; Zvirin et al. 2010) have begun to

shed light on the mechanisms underlying some of these processes and their role in pathogenesis.

Zvirin et al. (2010) used a GFP marked strain of Fom race 1,2 to monitor invasion of a susceptible melon cultivar Ein Dor. They reported that penetration of the epidermis, cortex and the xylem took place between 2 and 4 days post-inoculation (dpi). By day 11, the xylem was heavily populated with mycelium, and conidia were produced and germinated within the vessels.

Also, colonization dynamics of Ein Dor seedlings were compared with those of a genetically-resistant line, BIZ. It was observed that Fom colonized the resistant plant's vascular system, but the incidence of seedling infection was lower than in Ein Dor, suggesting stronger defense responses in BIZ expressed at the prexylem stage of infection. It was observed that Ein Dor seedlings wilted and died at 14 dpi, whereas BIZ plants remained symptomless and were visibly healthy even at 21 dpi.

To explore the possible molecular basis of melon resistance to Fom, the expression of three representative defense genes, hydroperoxide lyase (HPL), phenylalanine ammonia lyase-1 (PAL) and chitinase-1 (CHI), were compared between the resistant line BIZ and the susceptible cultivar Ein Dor. The transcript levels of the selected genes were compared, using real-time PCR, in the hypocotyls of resistant and susceptible seedlings before infection, and at 1 and 3 dpi by Fom race 1,2 (Zvirin et al. 2010). Both constitutive and induced differences in all three transcripts were detected between the resistant and the susceptible melon genotypes. The authors speculated that the differential expression of these genes could indicate their mechanistic importance in the defense against Fom. However, they did not provide clear evidence whether the three defense genes are indeed crucial for mounting an effective defense, or merely represent *markers* of the response.

To our knowledge, appropriate internal controls for gene expression studies during melon/Fusarium interactions have not been identified to date. A transcriptome approach using cDNA-amplified restriction fragment length polymorphism (AFLP) analysis has allowed the identification of differentially expressed transcripts associated with the infection process and the resistance response in the binomial interaction between the melon and Fom. Thus, Lotan-Pompan et al. (2007) identified using suppression subtractive hybridization and cDNA-AFLP, seven genes whose expression is associated with resistance to Fom race 2 following trifluralin herbicide treatments. Furthermore, the authors demonstrated that expression of four stress-related and up-regulated genes was enhanced when the plants were subjected to salinity stress, suggesting that trifluralin induces a general stress response which protects the plant against Fusarium wilt. Furthermore, 75 cDNA fragments with differential expression profiles between the races 1 and 1,2 of Fom were identified (Sestili et al. 2011).

16.2.5 Molecular Methods for Fom Detection and Quantification

Identification of Fom has been based almost exclusively on the isolation of the pathogen from symptomatic plants and inoculation of healthy plants of the same and/or related species. The fact that Fom is host-specific gives a generally reliable identification using this method. It is, however, laborious and time consuming. Because the selective medium is not species-specific or strain-specific and formae speciales of *Fusarium oxysporum* are morphologically identical, distinguishing between them in culture is not possible.

During the last decade, new DNA-based technology, such as polymerase-chain-reaction (PCR) assays, has been developed to support and replace morphology-based identifications of Fom (Validov et al. 2011; Zhang et al. 2005). However, most of these studies present difficulties pertaining to the development of molecular diagnostics at the formae speciales level (Validov et al. 2011). It is due to the polyphyletic feature of the majority of the formae speciales, that make the development of PCR primers at the forma specialis level difficult (Baayen 2000).

Generally, molecular identification of plant pathogenic fungi is based on the detection of polymorphisms in ubiquitously conserved genes, like translation elongation factor 1α (TEF-1α), ribosomal RNA, calmodulin and beta-tubulin (Lievens et al. 2008). The use of universal primers that anneal to conserved sequences flanking variable domains within these genes offers the possibility of detection and identification of genus/species/strain of fungi (Lievens et al. 2008; Seifert and Levesque 2004). However, these housekeeping genes do not generally reflect sufficient sequence variation for discriminating subspecies grouping like formae speciales and races (Lievens et al. 2008).

Therefore, additional strategies have been exploited to identify selective target sequences from genomic regions of unknown coding function such as random-amplified polymorphic DNA (RAPD) technology (Williams et al. 1990). Markers identified with this approach can be used to design specific sequence-characterized amplified region (SCAR) primers (Paran and Michelmore 1993) that specifically amplify the selected markers resulting in a robust identification assay (Larsen et al. 2002).

Using this approach, an SCAR marker, derived from a RAPD, was successfully identified for Fom detection (López-Mondéjar et al. 2012). The pair specific primers FOX-S/FOX-R of this marker was combined with a TaqMan probe and applied to amplify a 230 bp fragment from Fom isolates. This technique allowed a sensitive and rapid detection of Fom in melon plant material and substrate; as soon as 48 h after inoculation compared with 5–6 days required by the culture-dependent techniques. However, this TaqMan PCR system was not able to discriminate among Fom races.

Similarly, another PCR assay based on race-specific SCAR markers for the identification of Fom race 2 was developed which proved specific, sensitive, and reliable, regardless of the origin of isolates (Luongo et al. 2012). They identified the

marker RAPD OP-F15 that gave rise to a polymorphic repeatable band for Fom race 2 isolates. The polymorphic fragment was cloned, sequenced and converted to SCAR marker Fa15-F/Fa15-R which amplifies a single of 301 bp fragment specific to all isolates of Fom race 2. The specificity of this primer pair was tested against 8 isolates of Fom race 2 from different geographic origin, plus a total of 45 isolates of *Fusarium oxysporum* and other melon pathogens. No amplification was observed using DNA either from other Fom races or other *F. oxysporum* f. sp. as well as from the other melon pathogen used for comparison.

Currently, no molecular tools are available to discriminate among the four Fom races; although in tomato, assays for xylem-secreted effector-transcripts of *Fusarium oxysporum* f. sp. *lycopersici* were shown to correctly diagnose the fungal race (Lievens et al. 2009). It is expected that the knowledge that could be achieved by whole genome sequencing of further Fom strains could accelerate the development of new molecular markers. These markers could lead to better discriminate among the four Fom races.

16.3 Resistance to Fom Races 0, 1 and 2

16.3.1 Sources of Resistance

The basic requirement for the development of disease-resistant cultivars is the availability of dependable sources of resistance. In melon, the resistance to Fom was first reported by Messiaen et al. (1962) that found some resistant plants in French Cantaloupe Charentais genotypes and selected open-pollinated cultivars homogeneous for resistance to races 0 and 2, such as Doublon and Védrantais. They established that this resistance was conditioned by a dominant gene called Fom-1. Screening of the genetic resources led to the discovery of another independent dominant gene (Fom-2) in some accessions from the Far East (CM 17187) Messiaen et al. (1962). To date, these two genes have been extensively used in melon breeding and are already introduced to the majority of modern melon cultivars. However, it is desirable to have additional resistance sources available, because future adaptation of the pathogen could render specific resistance genes ineffective.

Therefore, several greenhouse, plastic tunnel and field evaluations were conducted to identify sources of resistance to Fom, beginning as early as 1976. Of 152 entries of melon germplasm tested for resistance to Fom race 2, Zink (1983), identified 32 cultivars that were highly resistant to this race. Additionally, Champaco et al. (1992) reported resistance to Fom races 0 and 2 in 6 melon varieties but failed to identify resistance sources to race 1. Pitrat et al. (1996) screened an extensive collection of *Cucumis melo* for resistance to Fom and found that 14.7 % of the 353 accessions tested were resistant to both races 0 and 2, and 13.8 % were resistant to races 0 and 1. In another study, Álvarez et al. (2005) found 16 new resistance sources to Fom races 0, 1 and 2 in a collection of 139 accessions from different geographical

origins. An exploratory comparison of all these studies revealed that, in melon, resistance to Fom races 0 and 2 is more frequent than that to Fom race 1.

16.3.2 Genetics of Resistance

Breeding for Fom resistance was initiated in France in the late 1960s using accessions of *Cucumis melo* (Messiaen et al. 1962). They described the dominant gene Fom-1 as responsible for resistance to Fom races 0 and 2, in the French cv. Doublon belonging to var. *cantalupensis*. The authors also identified the dominant gene Fom-2 conferring resistance to races 0 and 1 in the melon line CM 17187 collected from Indian and belonging to var. *acidulus*. Later, the gene Fom-1 controlling resistance to races 0 and 2 was described in some melon genotypes such as the gynoecious line WI-998-FR (Zink and Gubler 1986); the cultivars Honey Dew, Iroquois and Delicious 51 (Zink 1992) and the line Dulce (Danin-Poleg et al. 1999).

Another dominant gene, Fom-3, has also been reported as responsible for resistance to race 2 in a *cantaloupensis* melon line Perlita-FR from Texas (Zink and Gubler 1985). Allellism tests using F_1, F_2, F_3 and BC_1 generations from a cross between Perlita-FR, carrying the Fom-3, and Doublon, carrying Fom-1, inoculated with a race 2 isolate revealed the presence of susceptible individuals which is highly indicative of the presence of two genes (Zink and Gubler 1985). Today, it is still unclear whether this gene is identical to Fom-1. Risser (1987) affirmed that resistance in Perlita-FR is controlled by Fom-1 and the susceptible plants detected in the F_2 generation of the cross Perlita-FR x Doublon, used to test for allelism, could result from residual segregation occurring in the Perlita-FR parent used by Zink and Gubler (1985). In addition, Zink and Thomas (1990) described resistance to races 0, 1 and 2 in the monoecious melon breeding line MR-1 that was derived from an inbreed line PI 124111 (Thomas 1986). They established that the single dominant genes in MR-1 that confers resistance to races 0 and 2, and races 0 and 1 are the same genes or alleles of Fom-1 in cultivar Doublon and Fom-2 in line CM 17-187, respectively.

Similarly, Àlvarez et al. (2005) reported resistance to Fom races 0, 1 and 2 in the accession CUM-334 from Tajikistan belonging to var. *inodurus*. It was established that this accession carries the same genes or alleles of Fom-1 and Fom-2 as described in cv. Doublon and line CM 17-187, respectively (Oumouloud unpublished).

Recently, Oumouloud et al. (2010) reported a new recessive gene, fom-4, that confers together with Fom-1, resistance to races 0 and 2 in Tortuga, a Spanish var. *cantalupensis* accession. The inheritance studies and the molecular analysis demonstrated that Fom-1 and fom-4 assort independently. Recessive fungal resistance is likely to represent a mechanism distinct from that controlled by dominant resistance genes. Recessive resistance to fungal pathogens in crop plants has been characterized at the molecular level only in the case of the mlo gene in barley, which represents a defective negative regulator of constitutive defense (Buschges et al. 1997). Resistance under digenic control, involving two independent genes, one dominant

and the other recessive is not new in melon and was reported by Yuste-Lisbona et al. (2009) for resistance to *Podosphaera xanthii* races 1, 2 and 5 in line TGR-1551. Also this kind of mechanism was found in the snap bean cultivar Widusa against *Colletotrichum lindemuthianum* race 38 (Ferreira et al. 2003). As in the other *Fusarium oxysporum* f. sp., that have exclusively asexual reproduction and little potential for gene flow, Fom presents a low risk to overcome major resistance genes (McDonald and Linde 2002). However, this situation may change because of the extension of commercial cultivation of melons with the resistance controlled by Fom-1, and Fom-2. The use of different resistance genes will provide high levels of resistance to Fom races 0 and 2, and Tortuga may constitute a new alternative source of resistance. In addition, resistance controlled by more than one gene, both dominant and recessive, might increase its durability (Khetarpal et al. 1998). Furthermore, a better protection against race 2 could be achieved by combining different types of resistant genes, such as Fom-1, and fom-4.

16.4 Resistance to Fom Races 1,2

16.4.1 Sources of Resistance

Thus far, no gene that confers complete resistance to either race 1,2w or 1,2y has been reported, although partial resistance has been found. The first report of melon resistance to Fom race 1,2 was recorded by Risser and Rode (1973) from Indian accessions Ogon-9 and Piboule. Later, Pitrat et al. (1996) established that sources of resistance to race 1,2 are restricted to a few Far-Eastern accessions belonging to *Cucumis melo* ssp. *agrestis*. When screening melon accessions from different geographical origins for resistance to race 1,2, they found that only 3 % of the 271 accessions tested showed some resistance, all of them from the Far East.

The resistance to Fom race 1,2 described in accessions Ogon-9 and Piboule allowed breeding of partially resistant lines, such as cv. Isabelle (Risser and Rode 1973). The cv. Isabelle was crossed to Giallo di Paceco, an Italian landrace, and the resulting F_1 was subjected to parthenogenesis, haploid embryo rescue, and chromosome doubling. Two lines resulting from this process, Nad-1 and Nad-2, were selected for potential sources of resistance to Fom race 1,2w virulent isolate (Ficcadenti et al. 2002). The origin of the resistance in these doubled-haploid lines, which was greater than the resistance observed in the mother cv. Isabelle, may have developed from the homozygous status of resistance genes in Nad-1 and Nad-2, which allows full expression of the polygenic recessive resistance to Fom race 1,2 that is present within Isabelle. Polygenic resistance is based on minor genes, which may confer a higher level of resistance when all the genes are present together in a homozygous state. The use of the homozygous lines, such as Nad-1 and Nad-2, as breeding material in the genetic improvement can be extremely useful because it allows hastening the selection of cultivars with resistance to the pathogen. Therefore,

the employment of the genotypes in homozygous genetic background represents an effective strategy for maximizing resistance already present within the genotype.

Also, Herman and Perl-Treves (2007) reported a near complete resistance to Fom race 1,2 in the breeding line BIZ, and showed that BIZ definitely had a higher level of resistance than Isabelle. An F_1 hybrid, Adir, derived by crossing BIZ with a non-resistant counterpart, displayed good field resistance to Fom race 1,2. Adir also was used as a rootstock for the susceptible melon cultivar Ophir, and provided good protection against this race (Horev 2002). Additionally, Hirai et al. (2002) selected from Japanese melon landraces of var. *conomon* two melon cvs. Dodai No. 2 and Dodai No. 1 that showed partial resistant to the race 1,2y. They reported that both cultivars were graft compatible with major scion cultivars and effectively controlled *Fusarium* wilt of melon caused by race 1,2y when used as rootstocks in infested fields of productive areas in Hokkaido.

High resistance levels to Fom race 1,2 have been described in three Japanese melon accessions (Kogane Nashi Makuwa, C-211 and C-40) belonging to var. *makuwa*, and useful levels in one Russian (C-160) and two Spanish (C-300 and Mollerusa-7) accessions all of them from the var. *inodurus* (Oumouloud et al. 2009). Also, a partial resistance have been reported to Fom race 1,2 within a Portuguese accession BG-5384 belonging to var. *cantalupensis* (Chikh-Rouhou et al. 2010). Matsumoto et al. (2011) identified high-level resistance to race 1,2y in 34 of 76 accessions from 7 wild *Cucumis* spp. However, because of strong reproductive barriers to inter-specific crosses between melon and the wild *Cucumis* spp., this resistance could not be introduced into melon cultivars.

16.4.2 Genetics of Resistance and QTL Mapping

Resistance to Fom race 1,2 is complex and appears to be under polygenic control. Perchepied and Pitrat (2004) reported the mode of inheritance of resistance to Fom race 1,2 in the partially resistant line Isabelle using a RIL population derived from the cross between Isabelle and the susceptible cv. Védrantais. They confirmed that resistance to race 1,2 in Isabelle is under polygenic control, and established that the heritability of resistance was high (0.72–0.96), and the minimum number of effective factors controlling the resistance ranged between 4 and 14.

In a subsequent study the same population RILs was subjected to full marker-assisted analysis of quantitative trait loci (QTLs) (Perchepied et al. 2005). A total of nine QTLs associated with resistance to Fom race 1,2 (fomIII.1, III.2, III.3, V.1, V.2, VI.1, VI.2, XI.1 and XII.1) were identified and mapped, and together explaining 41.9–66.4 % of the total variation. Most of these QTLs appear to be recessive. The resistant alleles of seven QTL originated from the partially resistant parent Isabelle, whereas resistance alleles of two QTLs came from the susceptible parent, Védrantais. This agreed with the observed significant transgression towards susceptibility (Perchepied and Pitrat 2004). Besides, they provided experimental evidence of race-specific QTLs for resistance to race Fom 1,2 from the fact that the QTLs fomIII.1

and VI.1 were specific for the 1,2y pathotype, while fomV.2 and fomXII.1 were only identified following inoculation with the 1,2w pathotype. Thus, they interpreted this as supporting the assumption of Parlevliet and Zadoks (1977) that minor-gene-for-minor-gene interactions occur in pathosystems.

On the other hand five QTLs, fomIII.2, III.3, V.1, XI.1, and XII.1, were effective against both pathotypes 1,2y and 1,2w. Thereby, it was stated that partial resistance to Fom race 1,2 is governed by pathotype-shared loci, as well as by pathotype-specific loci. Perchepied et al. (2005) also co-localized QTL fomV.2 with the resistance genes Vat, which confers resistance to aphid colonization and virus transmission, and Pm-w for powdery mildew resistance, within a cluster of resistance gene homologs (Brotman et al. 2002; Garcia-Mas et al. 2001). In addition, QTL fomXI.1 was co-localized with the gene Fom-2, which confers resistance to Fom races 0 and 1. This gene has not been reported to contribute to race 1,2 resistance, therefore the loci implicated in resistance to Fom race 1,2 and races 0 and 1 may be different, but tightly linked. The presence of both quantitative and qualitative resistance genes in the same genomic regions suggests that QTLs may correspond to allelic variation of qualitative resistance genes with intermediate phenotypes (Robertson 1989).

Herman and Perl-Treves (2007) described a near-complete resistance to race 1.2 in the line BIZ, developed through selection of breeding material by breeders from the seed company Zeraim Gedera. The authors studied the inheritance of this resistance in the F_2 and backcross generations from the cross between BIZ and the susceptible line PI 414723. They found that segregation of resistance to race 1,2 in the F_2 and BC_1 generations fitted a 1:15 ratio of healthy plants in the F_2, and a 1:3 ratio in the resistant parent backcross; indicating that resistance to race 1,2 in BIZ is mediated by two complementary, recessive genes, designated fom-1,2a and fom-1,2b.

Herman et al. (2008) presented preliminary results of QTL analysis for resistance to Fom race 1,2 in the breeding line Biz using a set of 154 F_3 families derived from the cross between this line and PI 414723. They detected a major recessive QTL, designated fom1.2-a, that was derived from Biz and mapped at the linkage group II. Because this linkage group does not harbor any QTLs described by Perchepied et al. (2005) they assumed that Biz and Isabelle might carry different loci for resistance and not just different alleles in similar loci. Additionally, Chikh-Rouhou et al. (2011) studied the nature of Fom race 1,2 resistance in lines BG-5384, Shiro Uri Okayama, Kogane Nashi Makuwa and C-211, using F_2, and reciprocal backcross generations from the crosses between these accessions and Piel de Sapo a Fom race 1,2 susceptible melon line. They established that the resistance seen in all these accessions is polygenically inherited with relatively low heritabilities ranging from 0.48 to 0.59. Also, additive, dominance, and epistatic effects were significant in all crosses, which indicate that the resistance is under complex genetic control in the four accessions. Thus, the improvement of the character from these sources of resistance may be difficult and complicated to achieve through a standard selection procedure, which usually first exploits the additive gene effects. In another study, Foncelle et al. (2014) mapped five putative QTLs controlling resistance to

Table 16.1 QTLs for *F. oxysporum* f. sp. *melonis* race 1,2 resistance and linked markers

QTL	Locus	Primers	Predicted size (bp)
QTL1	Locus 3.1	5′-GCGTCATAGCGTACTTAGC-3′	322
		5′-ATTTGTTTTGCCATTTCTG-3′	
	Locus 3.2	5′-CCAAATCGAAACAAAAGTC-3′	303
		5′-TGTTAGATTTGTTGCAGGC-3′	
QTL2	Locus 6.1	5′-ACAAAATGGTAATGAAAACTTG-3′	237
		5′-AACAAGAAAGCTACCACGC-3′	
	Locus 6.2	5′-CCCATGAAAGAAAATGGAG-3′	189
		5′-TTCATCTTCCATCAAACCC-3′	
QTL3	Locus 7.1	5′-TAGCTTGAACTTCGTCCTG-3′	221
		5′-GAAGCGTACTCCCTATTGC-3′	
	Locus 7.2	5′-GGCAGTAAATGACCATGAC-3′	229
		5′-GGTGACGAACAAACTGAAG-3′	
QTL4	Locus 9.1	5′-TAGCAAACGACAACTAGGC-3′	329
		5′-GTGGAAAAGAGAGGAAAGG-3′	
	Locus 9.2	5′-CCCCTCTTATCTTTTCCTG-3′	279
		5′-CATCAAGAAGTCACGGAAG-3′	
	Locus 9.3	5′-CCAAAGTAAAAGTGAAGTCC-3′	147
		5′-CTTGAAATGAATTTGAGGTG-3′	
QTL5	Locus 10.1	5′-TTCTGATCAACGACGAAG-3′	246
		5′-GAAACAAAAGCCTCCATTG-3′	
	Locus 10.2	5′-ACCCACCATGCATTCTAAC-3′	251
		5′-GAGCCAGTTGGGGTTTTAG-3′	

Source: Foncelle et al. (2014)

Fom race 1,2 on melon chromosomes 3, 6, 7, 9 and 10 using an F7 RILs population derived from the cross between the resistant 03MFR001795 double haploid line and the Charentais type breeding line MFR0040308 which is susceptible to race 1,2 (Table 16.1). These QTLs were characterized by their additive and dominant effects. The positive additive values identified for all five QTLs meant that the 03MFR001795 parent carried the favorable alleles for these QTLs.

16.5 Characterization of Melon Accessions Resistant to Fusarium Wilts

The accessions in which some level of resistance to race 1,2 was found (Oumouloud et al. 2009), together with some accessions previously reported by Àlvarez et al. (2005) as resistant to races 0, 1, and/or 2 of Fom, (Table 16.2) were characterized for several agronomic traits and their genetic similarity was determined by using melon SSR markers, in order to assess the usefulness of these materials in breeding

Table 16.2 Some melon accessions characterized for several agronomic traits, their origins, botanical variety and their reaction when inoculated with *Fusarium oxysporum* f. sp. *melonis* races 0, 1, 2 and 1,2

Accession	Origin	Botanical variety	Reaction to *Fom* races[a]			
			0	1	2	1,2
Amarillo Manchado	Spain	*inodorus*	R	S	R	S
BG-4078	Spain	*inodorus*	R	S	R	S
Cum-241	Libya	*inodorus*	R	H	H	S
Banda de Godoy	Spain	*inodorus*	R	S	R	S
C-160	Russia	*inodorus*	R	S	R	PR
Amarillo Cáscara Pínta	Spain	*inodorus*	R	S	R	S
Maduro Amarillo	Spain	*inodorus*	R	S	R	S
Mollerusa-7	Spain	*inodorus*	S	S	S	PR
C-300	Spain	*inodorus*	H	S	H	PR
Piel de Sapo Monoico	Spain	*inodorus*	R	S	R	S
Cum-334	Tajikistan	*inodorus*	R	R	R	S
C-211	Japan	*makuwa*	H	H	S	PR
C-181	Japan	*inodorus*	S	S	S	PR
Amarillo Alargado	Spain	*inodorus*	H	S	H	S
C-40	Japan	*makuwa*	R	R	H	PR
CM-17.187	India	*acidulus*	R	S	R	S
C-87	Afghanistan	*conomon*	R	R	S	S
Tortuga	Spain	*cantalupensis*	R	S	R	S
Kogani Nashi Makuwa	Japan	*makuwa*	H	H	S	PR
Cum-355	Iraq	*cantalupensis*	R	H	H	S

Source: Oumouloud et al. (2009)

[a]R, PR, S and H indicate resistant, partially resistant, susceptible and heterogeneous phenotypes respectively

programs, aimed to the selection and breeding of resistant genotypes with high-yielding performance and qualitative traits accepted by the markets (Oumouloud et al. 2009). These authors reported that the resistance to races 0, 1 and 2 is scattered along all melon botanical types, whereas the high levels of resistance to race 1,2 was found only among accessions belonging to *Cucumis melo* ssp. *agrestis*, that appeared to be distant from commercial melon types both at molecular and morphological levels (Fig. 16.3).

16.6 Molecular Characterization of Resistance Genes Fom-2 and Fom-1

16.6.1 Characterization of Fom-2 Gene

The Fom-2 gene, conferring resistance to races 0 and 1 was originally identified in melon line CM17187 (Risser 1973; Risser et al. 1976) and was mapped to the linkage group XI (Perin et al. 2002). Fom-2 was cloned by a map-based cloning strategy

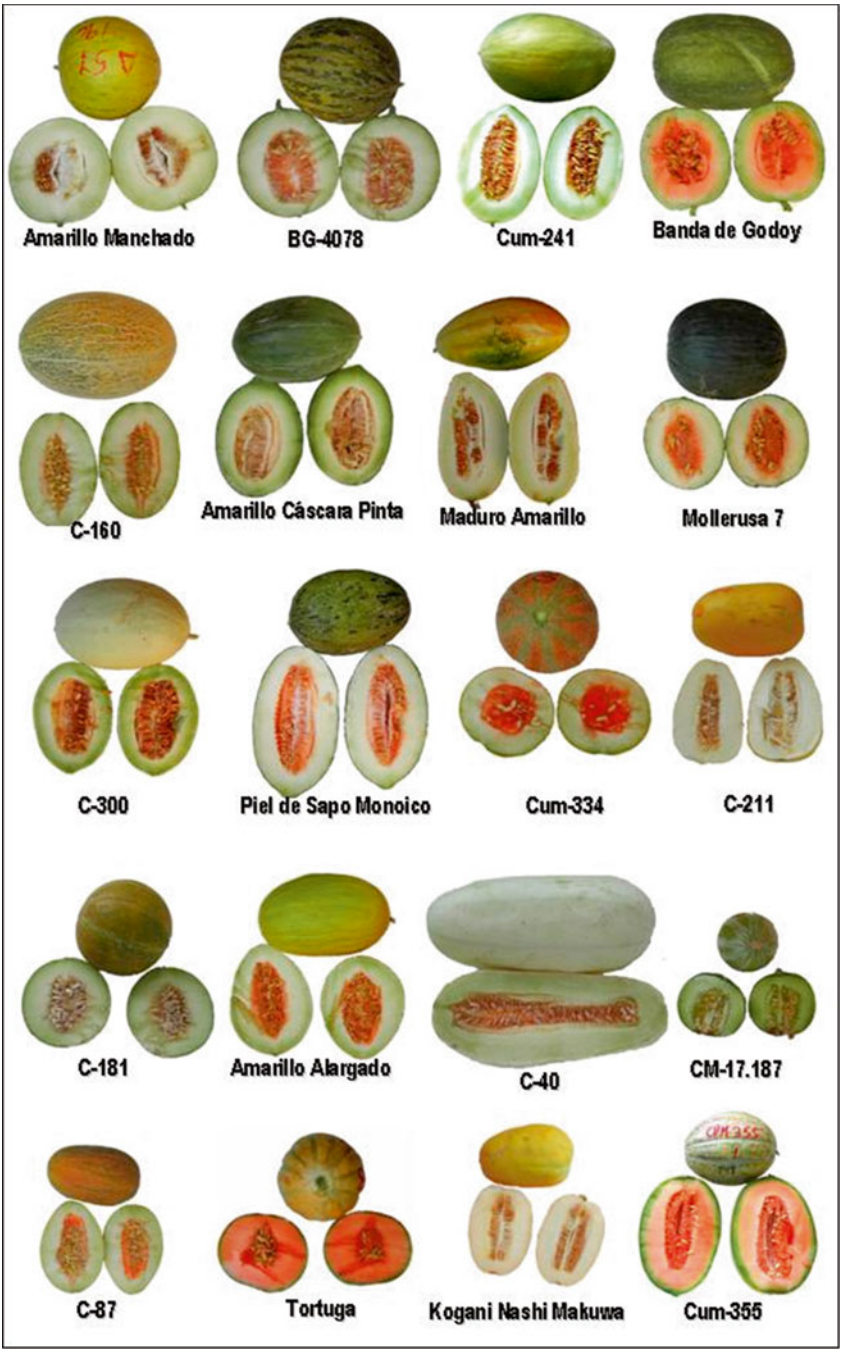

Fig. 16.3 Fruits of 20 *Cucumis melo* accessions, resistant to Fusarium wilt (Source: Oumouloud 2008)

(Joobeur et al. 2004). The isolated gene contains an uninterrupted open reading frame of 3 kb predicted to encode a polypeptide of 1073 amino acid residues that includes the different features of non-TIR NBS-LRR proteins. A large variation was detected within the LRR domain of this gene since 25 of 541 amino acids assessed differed between the resistant lines (MR-1 and PI 161375) and the susceptible varieties (Védrantais, Ananas Yokneam and Durango) analyzed (Joobeur et al. 2004). These results suggest that MR-1 and PI 161375 lines contain two different resistant alleles of Fom-2 gene.

Furthermore, Oumouloud et al. (2012) reported the cloning and sequencing of a partial LRR region of Fom-2 in 11 resistant melon accessions from various geographic regions. They identified three alleles of Fom-2 and their results revealed that the structure of the Fom-2 LRR domain is highly conserved, since 8 of the 11 resistant genotypes showed similar alleles to the resistant one characterized in the PI 161375 line. Conversely, PI 124111 was the only line that presented the same resistant allele previously described in MR-1. This could be explained by the ancestral relations between the two lines (Monforte et al. 2003), as the MR-1 breeding line was derived from PI 124111 (Thomas 1986). Finally, accession Cum-355 carried a novel resistance allele that differs from both PI 161375 and MR-1.

Additionally, the information generated from the Fom-2 LRR region sequences allowed systematic development of functional markers that were developed based on the nucleotide polymorphisms detected between the susceptible and resistant Fom-2 alleles (Oumouloud et al. 2012; Wang et al. 2011). In this context, Wang et al. (2011) reported two cleaved amplified polymorphic sequences markers (CAPS), representing allele-specific markers based on SNP in the LRR region of Fom-2 (Table 16.2). In a parallel study, Oumouloud et al. (2012) developed two simple and efficient SCARs, Fom2-R_{408} and Fom2-S_{342}, that represent a pair of allele-specific markers (Table 16.3). The two primer pairs can be combined in a multiplex PCR reaction, providing together a co-dominant marker. Such SCARs resulted in good identification of 27 resistant genotypes representing several melon horticultural types, enhancing the reliability and cost effectiveness of marker assisted selection for the Fom-2 gene.

Thus far, however, functional validation of the Fom-2 gene has not been reported. A preliminary study using transgenic roots of composite melon plants reported the expression of the Fom-2 promoter fragment along the vascular tissues (Normantovich et al. 2012). Using the same system to express the Fom-2 coding sequence in a Fom-susceptible background resulted in partial resistance in most, but not all experiments (Normantovich et al. 2012).

16.6.2 Characterization of Fom-1 Gene

The dominant gene Fom-1, controlling resistance to *Fusarium oxysporum* races 0 and 2, was reported in the old French cultivar Doublon (Risser 1973; Risser et al. 1976). Fom-1 was mapped at a distal part of the linkage group IX (formerly 5)

Table 16.3 Single-locus markers linked to Fom-2 gene

Marker name	Marker type[a]	Primers	Restriction enzyme	Reference
CAPS2	CAPS	5′- GGAAGTGAGGTGTTGAATT-3′	EcoR I	Wang et al. (2011)
		5′- TACACATTGGTCCGTTAGAC-3′		
CAPS3	CAPS	5′- AGACGTAGCATTGCTTCTCTAG-3′	Xba I	Wang et al. (2011)
		5′- TGGCATCCTTCAGCACCTTC-3′		
Fom2-R_{408}	SCAR	5′- GAGAAATTTGCAATGGGTGG-3′	–	Oumouloud et al. (2012)
		5′- TTACACTATTATTGCTCAACTTGC-3′		
Fom2-S_{342}	SCAR	5′- ATGAAAAGAAAAGATAACGACGA-3′	–	Oumouloud et al. (2012)
		5′-ATTGCTCTAAGTTGATCATATTCTG-3′		

[a]SCAR sequence characterized amplified region, CAPS cleaved amplified polymorphic sequences

(Perin et al. 2002). Using RAPD or AFLP markers and the *bulked segregant analysis* (BSA) method (Michelmore et al. 1991) several molecular markers linked to this gene were identified (Table 16.4) (Brotman et al. 2005; Oumouloud et al. 2008, 2009; Tezuka et al. 2009). However, their usefulness in determining the Fom-1 genotype is somewhat limited since they do not separate all genotypes according to their resistance phenotype; instead, it seems that each marker will have application only in a specific melon genetic background.

In addition, the technique based on the cloning of resistance gene homologues (RGH) has also been described as an approach for tagging resistance genes since they are often arranged in clusters in the plant genome (Yu et al. 1996). Using this method, Garcia-Mas et al. (2001) have identified the first melon resistance gene homologue (MRGH21) linked to Fom-1. Subsequent studies established that the melon genomic region harboring Fom-1 contains a cluster of eight melon RGHs, and the BAC (BAC 31O16) encompassing this RGHs was sequenced and characterized (Van Leeuwen et al. 2005). Based on the BAC 31O16 sequence Tezuka et al. (2011) developed more DNA markers linked to Fom-1, nevertheless their usefulness was variety-dependent. The authors suggested that Fom-1 could mapped between C-MRGH13 and 62-CAPS markers, which delimit a 137.462 bp interval.

More recently, Brotman et al. (2013) isolated the Fom-1 gene by a map-based cloning strategy in a large mapping populations (1190 plants) derived from a cross between the resistant line Védrantais and the susceptible line PI414723, backcrossed to PI414723. The authors considered the MRGH9 as a candidate gene for the Fom-1 locus.

Further studies are required to elucidate the structure and expression of the Fom-1 gene, to develop molecular markers within this gene and to provide functional validation of the Fom-1 gene action.

Table 16.4 DNA markers linked to Fom-1 gene

Marker name	Marker type[a]	Primers	Restriction enzyme	Reference
NBS1-CAPS	CAPS	5′-TATTGCTAAAGCTGTTTTCAAAAGCG-3′	Alw26 I	Brotman et al. (2005)
		5′-AACAAAAACTTTTCGATTTCCTAAGTT-3′		
62-CAPS	CAPS	5′-GGAGAAGATGCTAGAGCCATTC-3′	Nco I	Brotman et al. (2005)
		5′-AATCGGGCATCCTGTTTTGG-3′		
$SB17_{645}$	SCAR	5′-AGGGAACGAGTTGAGAGAGCTAGA-3′		Oumouloud et al. (2008)
		5′-CGAGGATTCTTAACTAGCATGGA-3′		
$SV01_{574}$	SCAR	5′- TGACGCATGGAATGAAATAAA-3′		Oumouloud et al. (2008)
		5′-GCATGGCCAAGGTCGAATA-3′		
$SV06_{1092}$	SCAR	5′-ACGCCCAGGTATCATATACACC-3′		Oumouloud et al. (2008)
		5′-ACGCCCAGGTTACGAAGTCA-3′		
CAPS2	CAPS	5′-CAATTTTGGTTTCTTTGGATGG-3′	Taq I	Tezuka et al. (2009)
		5′-TTTCGAGGTTAGAGGTTTGTCA-3′		
S-TAG/GCC-470	SCAR	5′-GAATTCTAGACTGAGCTTATAAACC-3′		Tezuka et al. (2009)
		5′-TTAAGCCTAAAAGGGAATGGCCCCC-3′		
C-TCG/GGT-400	CAPS	5′-TTCAAAATCAAAGGAAATGCAA-3′	EcoR I	Tezuka et al. (2009)
		5′-GGACCCAAACTTACCCTACACTT-3′		
618-CAPS	CAPS	5′- CTGGAGCCCAAATGAACAAAC-3′	Tfi I	Oumouloud et al. (2010)
		5′- GCTGGAGCATTCTAGTAATGAAA-3′		
S-MRGH9	STS	5′-GGTTGGCGATCTCACTGGAG-3′		Tezuka et al. (2011)
		5′-TTTACCAATTCCGCCCATCC-3′		
CAPS3	CAPS	5′-GTTGGAGATGTTCCCTTGGA-3′	Hae III	Tezuka et al. (2011)
		5′-ACCTGGCAACTTTGGTTTTG-3′		
C-MRGH12	CAPS	5′-CGTCGGGTATGTCTCCATCT-3′	Xmn I	Tezuka et al. (2011)
		5′-TGATGCTGCTGATGGACTTC-3′		
C-MRGH13	CAPS	5′-CCACCCATTCCCCATATTCC-3′	Taq I	Tezuka et al. (2011)
		5′-TGAGGAAGCAGGAGGGGAAC-3′		

[a]SCAR sequence characterized amplified region, CAPS cleaved amplified polymorphic sequences

16.7 Conclusions and Prospects

MFW caused by Fom is a devastating disease of melon worldwide. Two dominant resistance genes, Fom-1 and Fom-2, control resistance to Fom races 0 and 2, and 0 and 1, respectively. Both genes were introduced to many melon commercial cultivars. Partial resistance to Fom race 1,2 has detected predominantly in Far Eastern melon accessions belonging to *Cucumis melo* ssp. *agrestis*. These accessions are organoleptically far from the cultivated melons (Oumouloud et al. 2009). Genetic analysis revealed that resistance to Fom race 1,2 is complex and appears to be under polygenic control. Some biotechnological approaches, such as the development of double-haploid lines can be extremely useful (Ficcadenti et al. 2002), because they allow quick selection of genotypes with resistance to the pathogen, this would be favored if associated with some sort of marker-assisted selection. Taking into account that polygenic resistance is based on minor genes, these approaches could be an effective strategy for maximizing resistance, which may confer a high level of resistance when all the genes are present together in a homozygous state.

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Chapter 17
Viral, Fungal and Bacterial Disease Resistance in Transgenic Plants

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Abstract Continuing attention is being devoted to the development of substitute strategies in plant-disease management and reducing dependency on synthetic chemicals. Viral, fungal and bacterial diseases are unquestionably the most versatile for environmental adaption and in the destruction of plant growth. Among the strategies, resistance breeding has generated proven data and been exploited in depth. However, conventional methods alone are not sufficient to control the novel races of viral, fungal and bacterial pathogens in crops due to a scarcity in required crop variations. The current situation encourages the search for variation against biotic stress through identification of genes across species. Over the last two decades, significant efforts have been initiated in plant-disease management via genetic engineering. In addition, several molecular techniques have emerged to disentangle multifaceted plant-pathogen systems and associated disease-resistance candidate genes. Besides describing many promising candidate genes from viruses, fungi and bacteria, numerous plant disease-resistance genes have been identified and evaluated in crop improvement programs by transformation. Advancement in plant transformation techniques enables transferring useful genes for the rational creation of disease-resistant plants. Success has been achieved in transgenic crops against various diseases of important crop plants. This chapter describes genetically engineered plants and their resistant to viral, fungal and bacterial pathogens.

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17.1 Introduction

Global warming, the human population explosion and shrinking arable lands are among the major issues which require a sustained solution to be able to feed the nine billion world population by 2050. Plant pathogens frequently alter their behavior to survive in changing environment (Fisher et al. 2012). Therefore, efforts made so far to control plant diseases are inadequate. Chemical pesticides in use are rapidly losing their potency against mutating plant pathogens. Furthermore, uncontrolled use of pesticides has raised serious concerns of pathogens developing resistance to them. (Bosch et al. 2014). These challenges could be efficiently met with biotechnological inventions. This review describes plant genetic engineering efforts to find novel strategies for plant disease management (Collinge et al. 2010; Saharan et al. 2008).

Advancements in transgenic technology have great potential to benefit farmers, consumers and agro-food industries worldwide. Under the specter of global warming, disease control is the primary focus in the coming years for sustained crop yield and quality under the stress of novel races of pathogenic fungi, bacteria and viruses. Since the Green Revolution, quantifiable successes have been achieved in disease resistance breeding programs by the transfer of resistant loci from wild relatives to commercial cultivars (Bruehl 1991; Gómez et al. 2009). However, pathogens continuously evolve mechanisms to overcome resistance in crop plants. The breakdown of resistance is common and an unstoppable event which induces broad epidemics in the concerned crops (Table 17.1) (Fry 1993; Talbot 2004). Pathogens have many components that promote their proliferation, establishment and the spread of disease in crops (Gururani et al. 2012; James 2003). Hence, before starting any exercise to deal with the novel pathogens, a thorough knowledge of the complexity of plant-pathogen interaction must be investigated (Gururani et al. 2012; Jackson and Taylor 1996). Development of disease-resistant crops has been stimulated by the inputs from the genomics and proteomics of plants and pathogens (Chern et al. 2001; Peremarti et al. 2010; Ramonell and Somerville 2002; Sanseverino et al. 2010). In this chapter, we critically review the basic understanding and advances in developing disease resistant transgenic crop plants.

17.2 Virus Resistant Transgenic Plants

A number of accounts of viral disease-resistant crops have been confirmed since the first report of a virus-resistant transgenic plant (Table 17.2) (Fitchen and Beachy 1993; Galvez 2012; Galvez et al. 2014; Kumar et al. 2012; Powell-Abel et al. 1986; Prins et al. 2008). The genetic engineering of virus-resistance crops has been

Table 17.1 Potential of present and future diseases of some commonly distributed crops

Disease	Distribution
Viral disease	
African cassava mosaic	Wide spread in Africa, Asia and America
Bunchy top of banana	Destructive in Asia, Australia, Africa and Pacific Islands
Bean golden mosaic	Caribbean Basin, Central America and Florida
Rice tungro disease	Severe crop losses in Southeast Asia
Fungal disease	
Downy mildew of corn and sorghum	Rapid distribution out of Southeast Asia
Late blight of potato and tomato	Emergence of new virulent races spreading worldwide
Karnal bunt of wheat	Severe crop losses in Middle East, USA and Asia
Sugarcane rust	Destructive in America and Africa
Chrysanthemum white rust	Important in Europe, Asia and USA
Citrus black spot	Severe in Central and South America
Bacterial disease	
Bacterial leaf blight of rice	Destructive in Japan, India and wide distribution
Cassava bacterial blight	Severe in Africa, America and Asia
Bacterial wilt of banana	Destructive in America and Africa

Source: www.plantwisr.org/knowledgebank/searchresult

comprehensively elevated by sequencing, isolation and cloning of a number of key genes of viruses. This along with associated advances in genetic transformation of a number of crops has opened up the possibility of an entire new approach in genetic engineering toward controlling plant-viral diseases (Young 2000).

17.2.1 Pathogen Derived Resistance

Pathogen-derived resistance (PDR) refers to the resistance obtained from a pathogenic virus. Therefore, the whole gene or a part of its sequence isolated from pathogenic virus is transferred to the susceptible plants to obtain resistance.

17.2.1.1 Viral Protein Mediated Resistance

Viral protein mediated resistance is a type of PDR exhibited when a transformed plant produces viral protein (coat protein, replicase protein and movement protein) that interferes with the life cycle of the invading virus. This type of resistance is further divided into three groups (a) coat protein mediated resistance (CPMR), (b) replicase mediated resistance (Rep MR) and (c) movement protein mediated resistance (MPMR).

Table 17.2 Virus resistance transgenic crops

Candidate PDR genes	Viral disease	Host plant	Targeted gene	References
Coat protein mediated	Ring spot virus (PRSV)	Papaya (*Carica papaya*)	PRSV-CP	Wani and Sanghera (2010)
	Zucchini yellow mosaic 2 Potyvirus	Squash (*Cucurbita maxima*)	ZYM2P- CP	Meng and Gubba (2000)
	Citrus psorosis virus (CPsV)	Citrus (*Citrus* sp.)	CPsV – CP	Zanek et al. (2008)
	Potato virus – X	Potato (*Solanum tuberosum*)	PVX- CP	Bai et al. (2009)
Replicase mediated	Rice yellow mottle virus (RYMV)	Rice (*Oryza sativa*)	RdRp	Palukaitis and Zaitlin (1997)
	Potato leaf roll virus (PLRV)	Potato	PLRV- Rp	Ehrifeild et al. (2004)
	Bean golden mosaic virus (BGMV)	Bean (*Phaseolus vulgaris*)	BGMV-Rp	Faria et al. (2006)
Movement protein mediated	Astobra, Caulimo, Nepo virus	Tobacco (*Nicotiana tabacum)*	MP	Cooper et al. (1995)
	Tobacco mosaic virus (TMV)	Tobacco	MP-P^{30}	Prins et al. (2008)
Post transcriptional gene silencing (RNAi)	Potato spindle viroid	Tomato	SiRNA of transformation vector	Schwind et al. (2009)
	African cassava mosaic virus (ACMV)	Cassava (*Manihot esculenta*)	Rep/AC-1	Vanderschuren et al. (2009)
Satellite RNA mediated	Cucumber mosaic virus (CMV)	Tomato	HV-CMV	Cillo et al. (2004)

17.2.1.2 Coat Protein Mediated Resistance

Coat protein mediated resistance (CPMR) is a type of transgenic virus resistance crop plants have developed by exploiting coat protein (CP) encoding sequences (Anna et al. 2002; Ferreira et al. 2002; Lehmann et al. 2003; Makeshkumar et al.

2002; Mundembe et al. 2009; Nomura et al. 2004). Appropriate CP sequences are isolated from the concerned virus genome with certain modifications, and transferred with regulatory sequences to target plants. Compared to control plants, resistance is observable in transgenic plants in the form of delayed appearance of symptoms as well as by reduced virus titer. Generally two mechanisms have been established for depicting CPMR (Beachy and Philos 1999). First, *recoating of invading viruses*, which describes how an expressed CP subunit recoats the invading genetic material of a virus. A recoated virus genome is incapable of exploring −ve RNA for reverse transcriptase. In the case of + ve RNA, the virus does not have access to host ribosome for viral protein synthesis. Thus invading viruses cannot multiply and therefore cannot infect the plants. The second mechanism, *blocking the receptors in transgenic cells*, could be described as various subcellular components acting as receptors or uncoating the site for invading viruses. The CP subunit expressed through a transgene, binds to receptors and prevents the association of virions with the receptor, thus making it unable to penetrate the plant. The transformed tobacco expressing TMV CP subunit not only expressed CPMR against tobacco mosaic virus, but also against the closely-related virions. This might be explained through significant homology in gene encoding CP subunits of two different viruses. Other strategies can be adapted to increase broad spectrum CPMR via multiple gene transformation for different CPs and the searching of homology sequences in gene encoding CP subunits. Specific mutation in CP coding sequence translated in transgenic cells produce defective subunits that have more inter subunit interaction and lead to aggregation of subunits with virus coded CP. Field performance of transgenic papaya and squash made CPMR a prime choice to integrate resistance in other economically-important crop plants. Tomato, cucumber, watermelon and potato are some other important crops that have been successfully transformed with CPs to achieve resistance against viral diseases (James 2014). Freedom II is another commercially-released transgenic squash which affords resistant to zucchini yellow mosaic (Meng and Gubba 2000; Gubba 2000). Similarly, in citrus introduction of a CP gene against the citrus psorosis virus (CPsV) was reported to be successful by Zanek et al. (2008). CP-transgenic papaya, namely Sun up and Rainbow, were the first such commercialized fruit trees in Hawaii (Wani and Sanghera 2010).

17.2.1.3 Replicase Mediated Resistance

In replicase mediated resistance (Rep MR), viruses need a replicase enzyme to perform replication of their genetic material in the host cell. The origin of Rep MR can be explained by the fact that mutated or truncated replicase express in host plant and impede replication of virus genetic materials. Therefore, a truncated replicase encoding gene has been tried in many crops for viral resistance. Rep MR acts at two levels, one at the transcriptional level by interfering RNAs and the other at the translational level by interfering with truncated Rep protein (Lawson et al. 2001). Indeed, it is still not clear which mode of Rep MR is acceptable universally. This ambiguity is due to the contradictory reports asserting the presence of truncated rep protein or

the presence of Rep RNA species in host cytoplasm. Transgenic rice expressing the RdRp of rice yellow mottle virus (RYMV) proved to have stable resistance to RYMV strains (Palukaitis and Zaitlin 1997). C1 gene encoding Rep from tomato yellow leaf curl Sardinia virus (TYLCSV) confers resistance to viral disease in *Nicotiana benthamiana* and tomato plants (Brunetti et al. 1997). Similarly, in potato, the complete sense PLRV replicase gene provided resistance to potato leaf roll virus (PLRV) (Ehrifeild et al. 2004). Transgenic tomato carrying a truncated replication associated protein gene of tomato yellow leaf curl virus-Israel (TYLCV-Is [Mild]) conferred resistance to TYLCV-Is. *Phaseolus vulgaris* carrying the rep gene of bean golden mosaic virus (BGMV) also manifested resistance to BGMV (Faria et al. 2006). However Rep MR showed a relatively narrow spectrum of resistance, i.e. resistance manifested only for the particular virus race from which the transgene was isolated. Hence, research on Rep MR in crop plants has not been further exploited.

17.2.1.4 Movement Protein Mediated Resistance

Movement protein mediated resistance (MPMR), as the name implies, has as its proposed function to facilitate movement of nucleoprotein and/or viral particles, intercellular/intracellular, through plasmodesmata and tubules. Movement proteins (MP) and virus together make a complex of 1.5–2.0 nm diameter which can easily pass through plasmodesmata (Citovasky et al. 1992). Transgenic plants expressing MP showed delayed infection with mild symptoms of viral disease. This strategy also manifested a broad spectrum resistance as the dysfunctional MP-tobacco plants interfered with the systemic spread of distantly-related and unrelated viruses such astobra-, caulimo- and nepo-viruses (Cooper et al. 1995). It appears that two distinct plasmodesmatal transportation mechanisms are utilized. The first is involved in increasing the size exclusion limits of plasmodesmata during localized trafficking of MPs. The second involves large tubular structures composed of MPs that appear to facilitate the movement of viral particles through enlarged plasmodesmata (Jackson and Taylor 1996). Tobacco plants engineered with P^{30} MP of TMV (lacking N- terminal amino acids), showed delayed appearance of infection and symptom of disease. The expression of dysfunctional or mutated MP genes has reported the broader resistance, compared to CP/Rep mediated resistance (Prins et al. 2008).

17.2.1.5 Viral RNA Mediated Resistance

Viral RNA mediated resistance (VRMR) relates to the fact that most of the disease-causing plant viruses have a RNA genome that encodes all essential proteins viz. movement proteins, coat proteins, replicase proteins etc. Previously, it was assumed that over-expression of one or more structural or functional proteins in a normal or a dysfunctional state in transgenic plants would confer protection against the virus at protein-level interaction. Several examples have justified the above statement

(discussed in CPMR and Rep-MR) whereas in several others the above statement is not true. So transgene appears to have confirmed resistance through its mRNA rather than by its encoded proteins (Jianping et al. 2001; Jiunn et al. 2003; Khaled et al. 2002; Nomura et al. 2004). Hence, the phenomenon produced from the results of further study is known as viral RNA-mediated resistance.

Lindbo and Dougherty (1992), in experiments with the transgenic tobacco expressing CP gene, did not find a considerable concentration of CP, but reported CP transcripts in cytoplasm which provided considerable resistance against tobacco etch virus. Jiunn et al. (2003) carried out molecular analysis of nine selected transgenic lines of papaya harboring ring spot CP gene and found it to exhibit different levels of resistance. The analysis revealed that the expression level of the transgene is negatively correlated with the degree of resistance. This finding suggests that the resistance is manifested by a RNA-mediated mechanism. Baulcombe (1996) reported several VRMR characteristics which help to understand the complicated phenomenon of virus resistance. Doughorty and Parks (1995) provided considerable insight into VRMR and proposed that transgene mRNA in virus-resistant plants induce degradation of RNAs with the same or complementary sequence within cytoplasm which has arrived from infected virus. This attractive hypothesis has received much support in recent years. As a result, a well-established phenomenon of gene silencing known as post transcriptional gene silencing (details in PTGS)/co-suppression/antisense suppression/VRMR/RNA interference was given. However, instead of adapting the traditional VRMR, they found that design of an RNAi system in crop plants has more versatility against viruses (Galvez et al. 2014).

17.2.1.6 Post Transcriptional Gene Silencing/RNA Interference

Post transcriptional gene silencing and RNA interference (PTGS/RNai) is another strategy to create viral disease resistance in plants. In cross-protection, an initial viral infection generates small interfering RNAs (siRNAs) species which provide immunity to further viral attack. These siRNAs have sequence homology with infecting viral genetic material. Therefore, siRNAs commence an RNA complex pathway to viral genetic material which is a favorable substrate for endogenous RNA degrading enzymes. As a result the virus cannot proliferate in the host (Doughorty and Parks 1995; Galvez et al. 2014; Kubota et al. 2003). Despite being elicited by homologous RNA species, RNA interference is also triggered by self-complementary hairpin RNAs. This cruciform structure is a very favorable substrate for RNAi enzyme machinery. As a consequence, a large number of siRNA populations have emerged to act on complementary RNAs species in the cytoplasm.

RNAi technology has been exploited through transgenic-mediated synthesis of siRNAs (Ghildiyal and Zamore 2009; Leibman et al. 2011; Wang et al. 2010). In this strategy, key conserved sequences of the viral genome are used in designing a hairpin RNA transformation vector which has inverted repeats separated by non-coding sequences. These inverted repeats of the hairpin RNA transformation vector produce hairpin RNAs. These hairpin RNAs are further subjected to DICER and

RISC (RNA inducing gene silencing complex) enzymes for subsequent production of siRNA and further degradation of the target viral genome in the host cytoplasm. Transgenic tomato plants exhibited resistance against potato spindle viroid through siRNA using a similar transformation vector (Schwind et al. 2009). In another report, the engineered transgenic cassava plants showed resistance to African cassava mosaic virus (ACMV) by expressing dsRNAs. Transgenic cassava lines with high levels of AC1-homologous small RNAs have ACMV replication associated with protein coding sequence imparting Rep/AC1-homologous hairpin double strain immunity (Vanderschuren et al. 2009).

17.2.1.7 Satellite RNA Mediated Resistance

Certain RNA sequences packed with a viral genome cannot replicate, move and pack independently but require assistance from viral genome sequences called satellite RNA (Lin et al. 2013). A viral genome which helps satellite RNA to perform its function is known as a helper virus (HV). Some strains of CMV encapsulate the satellite RNA in addition to their own function of coding messenger RNA. CMV satellite RNA depends on its HV CMV for their essential functions. A very good example of using multiple or partial copies of CMV satellite RNA is to display reduced symptoms against CMV in tomato transgenics (Cillo et al. 2004). Little is known about the mechanism of satellite RNA mediated resistance but this has been explained by RNA gene silencing. In adopting this new concept of resistance, sufficient caution must be taken as there are chances of generating novel viral sequence *super pathogens* (Dempsey et al. 1998).

17.2.2 Non-pathogen Derived Resistance

Non-pathogen derived resistance (NPDR) refers to resistance obtained from a non-virus origin i.e. gene(s) derived either from plants or any other non-pathogenic sources.

17.2.2.1 Ribosome Inactivating Proteins (RIPs)

Ribosome inactivating proteins (RIPs) are specific N-glycosidases that eliminate a specific adenine from the sarcin/ricin loop of the 28S rRNA. These proteins are committed to arrest protein synthesis at the translocation step and are synthesized as pre-pro protein in plants and stored in cell vacuoles (Stirpe 2013). Their translation-inhibiting activity has been exploited against viral diseases. Studies have revealed that RIPs act on virus protein synthesis in the host plant cells and therefore the infected virions are not able to generate the protein for their multiplication. The pokeweed antiviral protein (PAP; RIP Type-1) coding gene expressed in transgenic tobacco shows a low level of resistance against many unrelated viruses. Besides its

resistant nature towards pathogenic viruses, it also has toxic effects on plants. However, a terminal deletion mutation in PAP has shown antiviral activity without causing toxic side effects to the host plant. Type-1 RIP from iris bulbs, called IRIP, has been transferred to tobacco. Molecular studies of the transgenic tobacco plants and characterization of purified protein have revealed that the recombinant IRIP from tobacco leaves has the same molecular structure as the native protein from iris bulbs. The tobacco transformants showed no apparent phenotypic side effects indicating that ectopically expressed IRIPs are not cytotoxic to tobacco cells. Antiviral activity and lack of cytotoxity of the expressed IRIP in transgenic tobacco renders IRIP an interesting and useful tool for the engineering of virus resistance (Baranwal et al. 2002; Desmyter et al. 2003; Wook et al. 2002).

17.2.2.2 Viral Protease Inhibitors from Plants

Viral protease inhibitors from plants, studied with respect to their viral structural and functional proteins, revealed the necessity to process their polyproteins for survival in host cells. Some groups of viruses, namely clostero-, nepo-, como- and potyviruses, require cysteine protease activity to process their nascent polyprotein for replication. The plant community expresses various protease inhibitors which impart natural resistance towards viruses. Transgenic tobacco expressing cysteine protease inhibitors from rice has been successfully tested against tobacco etch virus (TEV) (Gutierrez-Campos et al. 1999). Despite these encouraging results, this method could not be implemented where certain viruses did not require protein processing. In addition, it has been reported that cloned genes for viral protease inhibitors have deleterious effects on plant enzyme systems (Blandenvoorde et al. 2000).

17.2.2.3 Plant Antibodies

Plant antibodies (Av-plantibodies) represent an attractive approach to protect plants against pathogens and create plants that are endogenously resistant to pathogens. This can be achieved by using genetic engineering techniques such as expressing heterologous antibodies and antibody fragments for producing *designer* plants with viral resistance. These plant antibodies are known as *plantibodies*. Functional full-size antibodies and single-chain variable fragments (scFv fragments) can be targeted to different compartments of the plant cells. Cytosolic expression of specific scFv fragments can be used to protect plants from intracellular pathogens and to inhibit enzymes or hormones involved in the growth of viral pathogens. Extracellular targeting such as to the plasma membrane or retention in the endoplasmic reticulum gives high expression levels of correctly folded recombinant antibodies in plants. Targeting antiviral scFv fragments to plant cell membranes via heterologous mammalian membrane anchors has conferred resistance to tobacco mosaic virus. These surface expressions of the virus-specific scFv fragment may be a novel approach to shield the plant cell from an invading pathogen. Combining this strategy with cytosolically-expressed scFvs specific for conserved viral functional domains such

as movement proteins or replicase protein could provide an even more attractive route for generating virus-resistant plants. Recently, a cytosolical-expression system was used to achieve virus resistance based on the expression of scFvs against a conserved domain in a plant viral RNA-dependent RNA polymerase, a key enzyme in virus replication. The selected scFvs inhibits complementary RNA synthesis of different plant virus RDRP in vitro and virus replication in planta. Moreover, the scFvs are also bound to the RDRP of the distantly-related hepatitis C virus. T1 and T2 progeny of transgenic lines of *Nicotiana benthamiana* expressing different scFvs either in the cytosol or in the endoplasmic reticulum showed various degrees of resistance against four plant viruses from different genera. Virus resistance based on antibodies to RDRP adds another tool to the repertoire for combating plant viruses (Boonrod et al. 2004).

17.3 Fungal Resistant Transgenic Plants

Plant pathogenic fungi are considered the most versatile for environmental adaption and in the destruction of plant growth. Among the several approaches, genetically-engineered plants are assumed to impart resistance against fungal pathogens. Expression of antifungal compounds in transgenic plants has been a major approach to protect against fungal diseases and reduce the dependency on harmful synthetic fungicides (Wani 2010).

17.3.1 Antifungal Compounds

A wide range of antifungal compounds have been screened against fungal pathogens. Compounds which inhibit fungal growth are abundant in nature (Hegedüs and Marx 2013; Van Der Weerden et al. 2013). These antifungal compounds are natural sources of resistance in plants during various stages of development. Genes encoding such compounds for fungal-disease resistance are discussed below.

17.3.1.1 Chitinase and Glucanase

Chitinase and glucanase, the two most pivotal enzymes, have been studied in detail with respect to plant and fungal populations. Chitinase and glucanase catalyze the hydrolysis of two major structural components chitin and glucan, respectively, of the cell wall of many fungi. Chitinase genes have been identified from plants and micro-organisms and are broadly known as the PR-3 class of proteins. A number of reports of obtaining fungal-disease resistance through transformation of chitinase genes in many crops are available. The other enzyme glucanase is classified as a PR-2 class of proteins and are less studied compared to chitinase. These PR proteins

are inducible in nature and express under various conditions of pathogen attack, wounding, physico-chemical stress, etc. (Van Loon et al. 1994). Expression of chitinase and glucanase at low levels in transgenic plants has been a key issue. The low expression level of chitinase and glucanase transgenes depends on the host internal system viz. intracellular pH, cellular localization and environmental stress (Sela-Buurlage et al. 1993). Hence, isolation and selection of different chitinases and glucanases genes need to be screened to confirm their appropriate expression in a target crop. Chitinase of rice, lycopersicum, of fungal origin, has proved to be a good candidate in achieving resistance against fungal disease in certain crops (Tabei et al. 1998; Yamamoto et al. 2000). This has also proved that pyramiding of these two genes in transgenic crops promotes higher levels of resistance against fungal pathogen (Ram and Mohandas 2003; Wang et al. 2003). Studies have concluded that these enzymes hydrolyze the fungal cell walls and release oligo-N-acetyl glucosamines which function as elicitors for activation of a defense-related response in rice cells. In field trials, transgenic canola constitutively expressing a tomato endo-chitinase gene was found to exhibit increased resistance to fungal pathogens (Van Loon et al. 1994). In transgenic carrot, chitinase, β-1,3-glucanase in combination with AP24 gene gave rise to a broad spectrum fungal resistance (Stuiver et al. 2000). In general, tobacco, potato, sugar beet and rice have been transformed with chitinase gene and were found to be resistant to the fungus *Rizoctonia solani*. However, challenges still remain for those oomycetes, such as *Phytophthora* and *Pythium,* which do not contain chitin and, therefore, chitinases are ineffective (Datta et al. 2001).

17.3.1.2 Osmotin and Thaumatin-Like Proteins

Osmotin and thaumatin-like proteins (OLP and TLP) are important anti-fungal compounds. Most anti-fungal proteins found in plants share sequence homology with thaumatin, the sweet-testing proteins from the African shrub *Thaumatococcus daniellii* (Stintzi et al. 1991). These proteins have molecular masses of 22–26 kDa and are classified in the PR-5 family of pathogenesis-related proteins. These thaumatin-like proteins get induced upon microbial infection, oxidative stress, ABA, salicylic acid, methyl jasmonate, ethylene and certain wounding. Structural analysis has revealed their resistance to pH and heat denaturation by the presence of 16 cysteine residues which form 8 disulfide bonds. Broadly speaking, these PR-5 proteins induce fungal cell leakiness presumably through specific interaction with the plasma membrane which results in the formation of transmembrane pores. Transgenic potato plants expressing the tobacco osmotin (similar to thaumatin-like protein), which is basic 24 kDa pathogenesis-related protein that accumulate NaCl and regulate hormonal and environmental signals (Kononowicz et al. 1992). This showed delayed development of disease symptoms against *Phytophthora infestans* (Liu et al. 1994). Over-expression of rice TLP in rice itself, American ginseng, carrot and tobacco enhanced the resistance to various fungal diseases (Babu et al. 2003; Datta et al. 1999; Punja and Chen 2004; Velazhahan and Muthukrishnan 2004).

17.3.2 Small Cysteine Rich Proteins

Small cysteine rich proteins are usually small, cationic and amphipathic proteins having open-chain forms. The amphipathic structure with a α-helix and an anti-parallel β-sheet is highly conserved. The cationic hydrophobic residues are organized as segregate patches, resulting in a structure that is capable of forming ion channels through membrane bilayers. Furthermore, the compact and rigid structure is maintained by three or four disulfide bonds through cysteine residues. Following are two important small cysteine rich proteins which have an immense role in antifungal activities.

17.3.2.1 Defensins

Defensin, a plant antimicrobial protein, is a feasible natural candidate for fungal-disease control (Aerts et al. 2011; Carvalho and Gomes 2009; Kaur et al. 2011). Plant defensins are small cysteine-rich proteins consisting of 45–54 amino acids. They are synthesized naturally in plants, especially in seeds, and found in almost all plant organs. Although a majority of the defensins are secreted in the extracellular space, a few floral defensins are targeted to the vacuole. The best characterized defensins from radish Rs-AFP2 peptide shows enhanced resistance against the fungus *Alternaria longipipes* in transgenic tobacco. The remaining antifungal activity of the two groups, M-AMP2 and Ac-AMP2 peptides, have been proved in in vitro models only. A novel alf-AFP defensin peptide isolated from seeds of *Medicago sativa* displays robust activity against the fungal pathogen *Verticillum dahiliae* (Goa et al. 2000). The defensins peptide complex contains 4, 6 or 8 invariant cysteine residues which form intermolecular disulfide bonds. They contribute to the protection of seedlings against harmful microorganisms (analogous to the common fungicide coating of crop seeds) (Erik and Biezen 2001). However, defensins are generally not effective against bacteria (Broekaert et al. 1995).

17.3.2.2 Thionins

Thionins are small cysteine rich peptides (5 kDa) usually basic, very compact, amphipathic structures stabilized by three or four disulfide bridges and exhibit antibacterial and antifungal activities. Like defensins, the nascent protein chain of thionins is synthesized as pre-proteins and secreted into the vacuoles, intracellular spaces and cell wall. Naturally, thionins are expressed in the seeds, stems, roots of etiolated or pathogen stressed plant species. Notable results were obtained against *Fusarium oxysporium* f. sp. *matthiolae* in transgenic *Arabidopsis* expressing thionin peptide Thi2.1 (Epple et al. 1997). Accumulation of multicopy genes of the AMP group of thionins provides enhanced expression levels in transgenic crops (Isabelle et al. 2002). These small proteins are ancient systems of immune protection that

express during infection, inflammatory event and wound repair and their presence constitutes a key innate host defense against pathogens (Hancock and Diamond 2000). Thionins have a cationic charge which facilitates electrostatic attraction to negatively-charged surfaces of fungus. Their ability to assume amphipathic structures allows direct interaction with ubiquitous phosphoglycerol-lipids and their incorporation into microbial membranes. These peptides inhibit the growth of a broad range of the fungi at micro and molar levels in vitro, which is manifested by the changes in fungal morphology (i.e. reduced hyphal elongation and hyphal branching). Manipulation in attached signal peptide enables pathologists and molecular biologists to target these cysteine-rich peptides to specific sites of cells where a particular fungal attack predominates.

17.3.3 Plant Ribosome Inactivating Proteins

Plant ribosome inactivating proteins (RIPs) are RNA N-glycosidases that cleave a specific adenine residue in highly conserved sequence of 28S rRNA and inhibit the elongation factor eF-la to bind with ribosome. This irreversible modification blocks translation in ribosome assemblies. Some RIPs inactivate host-specific ribosome while others exhibit toxicity towards ribosomes from distantly-related species including animals and fungi (Stirpe et al. 1992). Based on structural diversity, plant RIPs are classified into three types (Table 17.3). As discussed in Sect. 17.2 above, these RIPs do not act on their own ribosome because they are targeted to vacuoles that sequester a certain development process. A RIP isolated from barley was shown to exhibit in vitro antifungal activity against a number of plant pathogenic fungi. Transgenic tobacco plant expressing isolated barley RIP gene under the control of inducible promoter showed increased resistance to *Rhizoctonia solani* (Logemann et al. 1992). An effective resistance was recorded in tobacco transgenics expressing

Table 17.3 Different types of ribosome inactivating proteins

RIP	Structure	Name and source
Type-1	Single chain	Pokeweed antiviral protein (PAP), pokeweed
	(N-glycosidase 29.5 kDa)	Pokeweed antiviral protein (PAPH), pokeweed hair root
	(N-glycosidase 11–30 kDa)	RIP 30, Barley
	(N-glycosidase 25 kDa)	RIP CCP 25, *Celosia cristata*
	(N-glycosidase 25 kDa)	IRIP, Iris bulbs
Type-2	Two chain	RIP, Caster
	(A chain- N-glycosidase)	
	(B chain-cell binding lectin)	
Type-3	Two dimmers of type-2	Various plants

Source: Saharan et al. 2008

a combination of RIP and chitinase (Chi-a) gene against *R. solani* (Jach et al. 1995). Rice blast caused by *Magnaporthe grisea* is one of the three major diseases that seriously affect rice production. Alpha-momorcharin (α-MC), a ribosome-inactivating protein (RIP) isolated from *Momordica charantia* seeds, has been found to exhibit in vitro antifungal activity (Qian et al. 2014). Further investigations are required into the transportation of RIP proteins and the way they bind with ribosome assembly (Stirpe 2013).

17.3.4 Phytoalexins

Higher plants synthesize a wide variety of secondary metabolites. Among them, phytoalexins play an important role in plant defense systems. Phytoalexins, a term originally coined by Muller (1958), are grouped under the class of plant antibiotics. These inducible antifungal and antimicrobial compounds are produced in plants after biotic or abiotic stresses. Their frequent accumulation is correlated to hypersensitive reaction (HR) of infected cells (Fig. 17.1). Phytoalexins are produced by healthy cells adjacent to localized damaged and necrotic cells in response to materials diffusing from the damaged cells. These diffused materials are known as

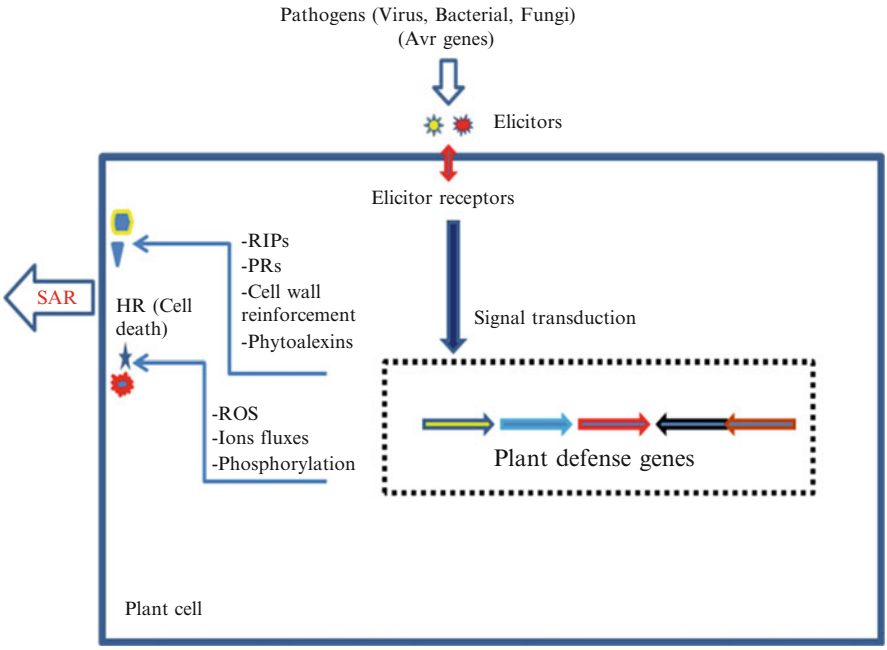

Fig. 17.1 Plant defense against pathogens

elicitors which trigger phytoalexin production. These elicitors play a key role in inducing the defense system of the plant cell. Phytoalexins accumulate around both resistant and susceptible necrotic tissues. Resistance occurs only when one or more phytoalexins along with other components reach a concentration sufficient to restrict pathogen development and therefore results in HR. The majority of biochemical and molecular evidence concerning the biosynthesis of phytoalexins has been obtained from the phenylpropanoid pathway which is also involved in lignin synthesis and to a lesser extent in terpenoid metabolism. The basic flavonoids skeleton is a derivative of two converging pathways, the acetate-mevalonate and shikimate pathways. These interconnected pathways are involved with various types of enzymes and their isomers. Leading enzymes which have a potent role are phenylalanine ammonia lyase (PAL), CoA ligase, chalcone synthase (CHS), chalcone isomerase (CHI) and stilbene synthase. PAL and CHS exist in isozymic form and are encoded by multigene families. Bean cells treated with elicitors revealed that CHI accumulates as a single polypeptide encoded by a single gene (Mehdy and Lamb 1987). The expression of grapevine stilbene synthase gene in rice plants has been shown to enhance disease resistance (Stark-Lorenzen et al. 1997). Similarly, resveratrol synthase and isoflavone methytransferase genes have been proved to enhance disease resistance in transgenic alfalfa (Hipskind and Paiva 2000). Alteration of phytoalexins through chemical engineering can be a way of stimulating more activity against fungal pathogens. Methylation of free hydroxyls has been shown to increase the antifungal activity of isoflavonoids. In addition, phytoalexins are often toxic to humans and/or animals. Consequently an inducible system may be applied for transgenic expression of phytoalexin gene(s) in plants (Großkinsky et al. 2012).

17.4 Bacterial Disease Resistant Transgenic Plants

Bacterial pathogens are responsible for numerous diseases in higher plants. Cereals, vegetables and fruits are common crops which are severely affected by bacterial diseases (Morgues et al. 1998). The development of bacterial disease resistant transgenic plants holds considerable promise to combat these pathogens.

17.4.1 Anti-microbial Protein

A large number of diverse, natural and cationic antimicrobial peptides (CAPs) have been discovered in recent years to strengthen resistance against bacterial diseases (Table 17.4). CAPs are the foremost active peptides among the antimicrobial peptides. These peptides fall in two classes: α-helical peptides, such as cecropines and maganins and β-sheet peptides, such as defensins, protegrins and lactoferrin (Huang et al. 2010). Amphipathic distribution of polar residues gives these peptides the

Table 17.4 Key achievement in transgenic production of antimicrobial proteins against bacterial disease

Transgenic protein	Crop	Resistance against	References
(Chimera protein) SP-cec B	Rice (*Oryza sativa*)	*Xanthomonas oryzae*	Sharma et al. (2000)
Msr A1 (Cecropin + chitinase chimera)	Potato (*Solanum tuberosum*)	*Erwinia carotovora*	Osusky et al. (2000)
MSI-99 (Melittin + cecropin) (synthetic protein analog)	Tomato (*Lycopersicon esculantum*)	Bacterial speck disease	Alan et al. (2004)
MB-39 (Melittin + cecropin) (synthetic protein analog)	Tomato	*Pseudomonas syringae* pv. *tabaci*	Jan et al. (2010)
Attacin E	Apple (*Malus malus*)	*E. amylovora*	Norelli et al. (1999)
Lysozyme	Potato	*E. chrysanthemi*	Rivero et al. (2012)
Lysozyme	Tobacco (*Nicotiana tabacum*)	*P. syringae* pv. *tabaci*	Nakajima et al. (1997)
Lysozyme	Potato	*E. carotovora*	During et al. (1993)
Lactoferrin (human)	Tomato	*Ralstonia solanacearum*	Lee et al. (2002)
Lactoferrin (bovine)	Pear (*Pyrus communis*)	*E. amylovora*	Malnoy et al. (2003)

ability to interact with the phospholipid membrane. This causes opening of the lipid bilayer and collapse of the trans-membrane electrochemical gradients leading to cell death (Bechinger 2004). Results have shown that these peptides are effective against plant pathogens (Alan and Earle 2002; Goyal and Mattoo 2014; Maroti et al. 2011).

17.4.1.1 Transgenic Expression of Cecropins

Cecropins are potent antimicrobial linear amphipathic peptides consisting of 31–39 amino acids residues and adapts α-helical structure on interaction with the bacterial membrane and induces pore formation. Natural cecropin and its synthetic analog (SB-37 and MB-39) gene have been introduced in tobacco plants and showed pathogen resistance (Huang et al. 1997). Norelli et al. (1999) transferred natural cecropin and its synthetic analog to enhance resistance against fire blight in Royal Gala apple. No effective resistance was observed in transgenic tobacco expressing a cecropins B gene against *Ralstonia solanaecarum* and *Pseudomonas syringae* pv. *tabaci* (the casual agent of tobacco wild fire). This was due to less expression of

transgene protein and degradation by host proteases. Therefore, to prevent cellular degradation of peptides by host peptidases, cecropins must be targeted to intercellular spaces. A transgenic rice plant carrying SP-cec B construct has been developed by fusing signal peptide (SP) of chitinase gene of rice which is known to direct the secretion of the gene product into the intercellular spaces in rice (Sharma et al. 2000). Targeted to intercellular space, cecropins sequestered from protease of the host plants provide a significant level of resistance against bacterial leaf blight in rice (Jan et al. 2010). Another cationic antimicrobial peptide called melittin, consisting of 26 amino acids, showed powerful haemolytic activity (Hancock and Diamond 2000). Osusky et al. (2000) reported broad-spectrum resistance to phytopathogens expressing an N-terminus-modified, cecropin-melittin chimera (Msr A1) in two potato cultivars. They modified the melittin peptide to reduce their toxicity towards haemolytic activity. Other small cationic peptides such as MSI-99, a synthetic analog of magainin II (MII), have been used in developing transgenic tomato plants for enhancing resistance to bacterial speck disease. Several MSI-99 expressing lines developed significantly fewer disease symptoms than controls. These results suggested that expression strategies providing continuous high expression of MSI-99 is necessary to achieve significant enhancement of plant disease resistance against bacterial speck disease (Alan et al. 2004). Co-operation between molecular modeling and engineered novel peptides provides a powerful tool to generate chimera peptides (Fox 2013).

17.4.2 Transgenic Expression of Lactoferrin Gene

Lactoferrins (~80 kDa) belong to a family of cationic iron-binding glycoprotein found in mammalian milk. A lactoferrin gene has been isolated, cloned and characterized from human and bovine sources. Its mode of action against bacteria is not only bacteriostatic but also bactericidal (Borther et al. 1989). The siderophores produced by many bacteria which are one of the virulence factors, allows bacteria to overcome the condition of iron limitations in host cells and has a protective effect against the toxicity of reactive oxygen species (Venisse et al. 2003). Thus decreasing iron availability in transgenic plants could be an attractive approach to limit bacterial survival in the host plant. Lactoferrin which has iron-chelating action could be a limiting factor of bacterial growth in transgenic host cells. Expression of the human lactoferrin gene in transgenic tobacco plants conferred increased resistance to *Ralstonia solanacearum* (Zhang et al. 1998). Similarly transgenic tomato exhibited partial resistance against bacterial wilt through the lactoferrin gene (Lee et al. 2002). Transgenic pear containing bovine lactoferrin cDNA conferred reduction in fire blight disease symptoms (Malnoy et al. 2003). Furthermore, medicinally-important ginseng and rice also produced high amounts of human lactoferrin. Besides their use for bacterial disease resistance, they are also used as food additives. Rice expressing lactoferrin may be a useful vehicle to introduce recombinant human lactoferrin to infant food (Kwon et al. 2003; Suzuki et al. 2003). Introduction of lactoferrin in transgenic cereals, fruits and vegetables

could be a new challenge to overcome bacterial diseases as well as make lactoferrin a hygienic food supplement (García-Montoya et al. 2012).

17.4.3 Other Antimicrobial Peptides

17.4.3.1 Attacins Expression in Apple and Pear

Attacins such as cecropins are small lytic peptides which show a substantial degree of resistance against bacterial pathogens. European apple cultivars are under great threat of bacterial fire blight caused by *Erwinia amylovora*. The attacins gene has been expressed in cultivars of apple and found less susceptible to the fire blight pathogen (Norelli et al. 1999). Royal Gala apple transgenic line TG138 containing attacin E under the control of pin II promoter had only 5 % shoot length blighted (SLB) as compared with 56 % SLB in non-transgenic Royal Galas and 37 % SLB in the moderately resistant Liberty control (Norelli et al. 1999). Transgenic cultivars Royal Gala, Galaxy and M 26 rootstocks expressing attacin LP under a constitutive promoter have also shown increased fire blight resistance (Aldwinckle et al. 2003). Besides apple, European pear (*Pyrus communis*) is also affected by *E. amylovora.* Here as well the transgene attacin E has been expressed against fire blight (Reynoird et al. 1999).

17.4.3.2 Transgenic Expression of Lysozymes

Lysozymes enzymes are widely distributed in nature and can be expressed transgenically. The human, chicken and T4 bacteriophage lysozyme cleaves the α-1–4 glycosidic bond of peptidoglycan in the bacterial cell wall. The T4 bacteriophage lysozyme cannot hydrolyze chitin, so human and egg white lysozyme has been used in many studies on phytopathogen resistant transgenic plants. So far, only a few research papers have appeared on the engineering of bacterial resistance in plants. One of the earlier reports regarding the transgenic potato expressing is the T4 bacteriophage lysozyme gene. The transgenic potato secretes lysozymes into the intercellular spaces, the site of entry and spread of the bacterium *Erwinia carotovora* (Rivero et al. 2012). Although expression levels of the transgene were found to be very low, the plants appeared to be less susceptible to *E. carotovora* infection than the control plants (During et al. 1993). A human lysozyme gene was transformed into tobacco and exhibited slightly fewer symptoms against the fungus *Erysiphe cichoracearum* and the bacterium *Pseudomonas syringae* pv. *tabaci* (Nakajima et al. 1997). Transgenic potato line R93 identified as less susceptible against black leg (*Erwinia chrysanthemi*), has been transformed with a chicken lysozyme gene through *Agrobacterium*-mediated transformation. However, these less-susceptible transgenic plants showed the same phenotype as the non-transgenic cultivars (Hirai et al. 2004). Rice cultivar Taipei 309 was utilized to evaluate the expression level of the human lysozyme gene under glutelin-1 promoter in maturing rice grain. At least 12 independently-transformed lines have been found with a significant level of lysozyme. The expression level of

lysozyme reached 0.6 % of brown rice weight, or 45 % of soluble proteins. Further segregation analysis has shown Mendelian inheritance with the same level of transgene protein expression. A similar study was conducted of transgenic rice expressing the human lysozyme in the endosperm, which revealed distorted trafficking and sorting of native storage protein in the rice endosperm and affected the expression of natural storage protein (Yang et al. 2003). A significant level of resistance in cultivars for commercial purpose is still to be achieved for bacterial diseases.

17.4.4 Strategies for Bacterial Virulence Factors

Developing strategies for bacterial virulence factors involve expressions of various compounds that help pathogenic bacteria to spread infection or carry out damage to host cells, they are known as virulence factors. These include the toxins, pectin enzyme, exo-polysaccharides, hormones, etc. Any mechanism expressed by a plant to inhibit bacterial pathogenicity or virulence factors can lead to resistance or reduced susceptibility. This knowledge has not been intensely investigated to develop strategies for engineering disease resistance (Baker et al. 2010). The wildfire disease of tobacco is caused by *Pseudomonas syringae* pv. *tabaci* which produces tabotoxin, a dipeptide toxin containing an uncommon β-lactum amino acid causing the chlorotic symptoms. The tabotoxin resistance gene ttr, encoding an inactivating acetylating enzyme from the same bacterium, was expressed at high levels in transgenic tobacco and successfully enhanced resistance to this bacterium. Further evaluation in the field of up to R7 progeny has confirmed a heritable resistance (Anzai et al. 1989; Batchvarova et al. 1998).

17.5 Exploiting Natural Plant Defenses

17.5.1 Transgenic Production of Elicitors

Transgenic production of elicitors has potential in natural plant defense. A variety of substances called elicitors are released by pathogens during infection of a host plant which are recognized by the plant as signal molecules and trigger defense mechanisms. In most cases, elicitors are synthesized by pathogens themselves but in a number of instances elicitors are produced as a result of a pathogen hydrolyzing the host cell walls. Pectate lyase (PL) enzyme is a major virulence factor of bacteria; it degrades the pectin component of the cell wall into unsaturated oligogalacturonates (OG) which are known to elicit a plant-defense response. A gene coding the isoenzyme pectate lyase-3 was transferred into potato and four PL3 transgenic lines selected over a period of 4 years exhibited enhanced resistance to *Erwinia* soft rot (Wegener 2002). Therefore, production of elicitors through transgenic means could be an effective strategy to enhance disease resistance (Fig. 17.2).

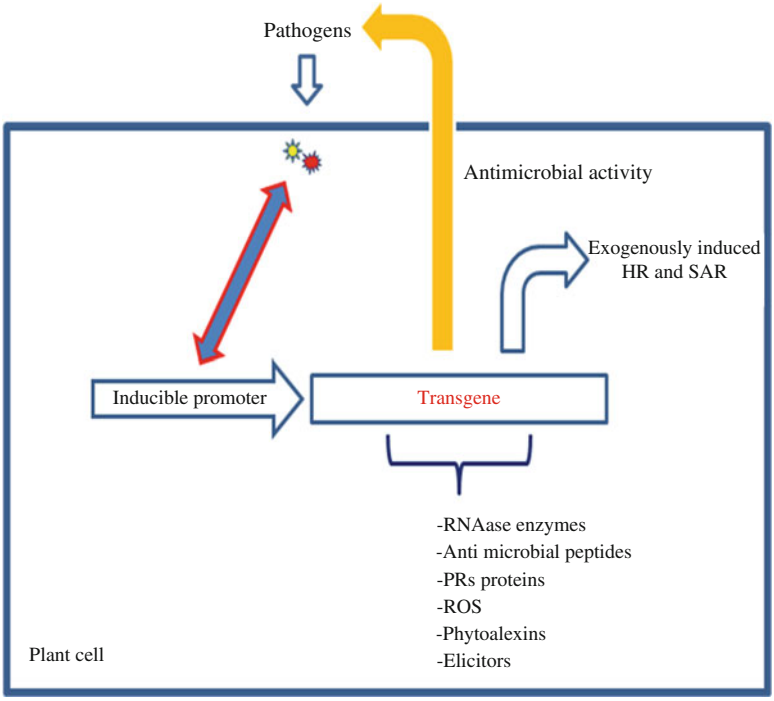

Fig. 17.2 Transgenically induced plant defense

17.5.2 Transgenic Production of Reactive Oxygen Species

Transgenic production of reactive oxygen species (ROS) also has potential in natural plant defense. The interaction of pathogen elicitors with host receptors activates a signal transduction cascade that involves other defense signals along with production of ROS. ROSs are directly related to enhancing the plant defense system, induce local hypersensitive reaction, systemic acquired response, etc. Enhancement of ROS production in plants could be an effective means to attain broad-spectrum disease resistance. The expression of the glucose oxidase (GO) gene in many plants induces hydrogen peroxide which results in an increased level of resistance to many bacterial pathogens and shows an increased level of hypersensitive response (Kachroo et al. 2003; Lee et al. 2002). High levels of GO expression in plant cells were associated with reduced growth of stem, root, less seed set and low seed germination. Hence, ROS expressing transgenes should be under precise control to protect the plant from a growth inhibitory effect from the transgene product (Fig. 17.2) (Murray et al. 1999).

17.5.3 Exogenously Induced Programed Cell Death

Exogenously induced programed cell death occurs when pathogens trigger a rapid and localized response in infected cells which kills them. This complex response is known as hypersensitive response (HR) or programed cell death (PCD). Exogenously induced programed cell death mimicking the natural HR is an alternative system to provide resistance to susceptible plants (Goyal and Mattoo 2014; Greenburg et al. 1994). However, this system may have deleterious effects on non-affected host cells. Therefore, there is need for a defined expression system which acts only on infected cells. A transgene responsible for inducing programed cell death could be attached to a pathogen inducible promoter so the transgene is expressed only in infected cells and the rest of the uninfected plant tissue not killed. This two-component system of transgenes (barnase and barstar) has successfully been expressed in transgenic potato plants against the fungus *Phytophthora infestans* (the causal agent of potato late blight). The availability of inducible promoters especially under pathogen infection is a major requirement to precisely control the transgene expression to avoid deleterious effects on healthy cells. In addition, controlled expression of transgenes saves energy which could be used in growth promotion of the host plant. Another approach which mimics the HR in plants to enhance resistance against pathogen attack is cloning of bacterio-opsin gene (bO) and proton pump. These genes are responsible for an accumulation of salicylic acid (a key chemical signal to systemic acquired resistance) and are inducers of an HR pathway. Cloning of the bO gene in tobacco increased resistance to TMV. The possibility of using a wound-inducible promoter to control the expression of bO did not develop spontaneous lesions. Nevertheless, under controlled laboratory conditions, they were found to be resistant to the pathogen. The activation of the defense mechanisms by the bO gene was not constitutive, and occurred in response to wounding or pathogen infection. Furthermore, wounding of transgenic tobacco plants resulted in the induction of systemic resistance to pathogen attack within 48 h. These findings provide a promising initial assessment for the use of wound-inducible promoters as new strategies to enhance pathogen resistance in transgenic crops by means of lesion mimic genes (Fig. 17.2) (Rizhsky and Littler 2001).

17.5.4 Cloning of R Gene for Disease Resistant Transgenic Plants

The R genes, naturally present in plants, are frequently used in breeding programs to produce disease-resistant transgenic plants. These genes are dominant, monogenic and provide resistance against one or few races of pathogen species. Race-specific resistance is explained by the gene-for-gene hypothesis proposed by Flor (1971) during his historical studies on the interaction between flax *(Linum usitatissimum)* and rust fungus *Melamspora lini*. According to this hypothesis, the plant

receptor (coded by the R gene) can recognize a pathogen-derived ligand (a product of avr gene) and ultimately convey signals to other defense-related genes for battling pathogens. Many techniques used to clone R gene(s) are still being pursued along with map-based cloning and transposon tagging (Tanksfey et al. 1995). Since the isolation of the first resistant gene, Hm1, about 20 R genes have been cloned. A general feature of the products of R genes is the presence of leucine rich repeats motifs, which are believed to be involved in recognition of avr gene products. Another protein motif is the nucleotide-binding site (NBS). This is assumed to be a regulatory switch for a signal transduction cascade (Kobe and Deisenhofer 1995).

R gene mediated genetically engineered plants have several attractive features for disease control. They have the natural mode of action that is homologous to the plant defense system and the concerted response can efficiently halt the growth of the pathogen. No input is required from farmers and there are no adverse environmental effects. However, R genes often become ineffective by co-evolving pathogens. Under selective pressure, the pathogens the avr gene evolves and become virulent in nature (thus coding mutated elicitors) and as a result the concerned R gene coded receptors cannot recognize pathogen infection. However, recent advances in structure and function of R protein and elucidation of new elements involved in downstream signal pathways provide a fertile field of the future scope of recombinant novel R genes (Wally et al. 2009).

17.5.5 R Gene Pyramids

The recent concept of cloning multiple R gene pyramids might provide strategies to overcome the above mentioned deficiencies. Transgenic use of the R gene, known as Bs2, cloned from pepper, has provided longstanding resistance against bacterial spot disease caused by the bacterium *Xanthomonas campestris* in tomato expressing NB-LRR (Thilmony et al. 1995). Other R genes cloned with potential use against fungal pathogens include the barley Rpg1 gene (Whitham et al. 1996) and tomato Ve1 and Ve2 genes (Strittmatter et al. 1996). The Rpg1 gene has provided remarkably durable resistance to stem rust for decades and Ve1 and Ve2 target *Verticillum* species that cause wilt in many different crops. The Ve genes can provide resistance to different *Verticillum* species and are functional in potato when expressed as transgenes. The Rpg1 and Ve genes have novel structural features that discriminate them from earlier R genes. Novel R genes can be used as prototypes to identify additional R genes to be used in genetic engineering. The phenomenon of *non-host resistance* exists when all varieties of plant species are resistant to all strains of a particular pathogen species. For example, *Arabidopsis* and tobacco are uniformly resistant to many microbes that plague crops (e.g. *Phytophthora infestans*). Recent studies have revealed that certain signal transduction components are responsible for non-host resistance (Bent et al. 1994; Salmeron and Staskawicz 1993). A similar reason was proposed for *restricted taxonomic functionality* which restricts the function of transgenes between distantly related species (Warren et al. 1997).

17.6 Measures for GM Crops Acceptance

Measures to promote GM crops acceptance is necessary in view of the current hue and cry against them. It is imperative to use technologies which decrease the risk associated with the blending of transgenes to a different genome. Efforts seek to implement more approaches of *non-pathogen derived resistance* to avoid the contamination of unrelated genes. In this connection, the most desirable approaches are the transferring of plant origin gene to crops viz. cloning resistance R gene, antimicrobial peptides, induction of HR and subsequent systemic acquired resistance (SAR). Artificial enhancement of the HR response could be the revolutionized option of broadened resistance towards the pathogens. Introduction of certain genes associated with SAR in crops could strengthen the natural immunity to combat future disease attacks in economically-important crops (Goyal and Mattoo 2014). Anti GMO lobbies have a number of concerns about transgenic crops like ethical issues, bio safety aspects etc. Owning to the benefits of candidate genes, the major concern are the selectable marker genes which may be toxic or allergenic to human beings; antibiotic selectable markers having wide clinical and veterinary applications. The marker gene could be transferred into microorganisms in the human and animal gut, which could render the microorganism resistance towards antibiotics. In addition to this, selectable markers have no function after selection and this exerts an extra load to the plant system. Therefore it is reasonable to consider removal of these extra genetic materials from the transgenic crops. Some successful methods are under current research which has the ability to remove these marker genes through co-transformation of a marker gene and the gene-of-interest followed by segregation, Intra-genomic relocation of transgenes via transposable elements, removal of the selectable marker gene after the selection procedure via site-specific recombinases and novel zinc finger nucleases are some of the methods could be used to remove selectable marker gene from transgenic crops (Tuteja et al. 2012).

17.7 Conclusions and Prospects

A major obstacle in accelerating transgenic technology against crop diseases is the lack of defined studies of plant-pathogen interaction at the molecular level to identify the resistance product and its genetics. Furthermore, the lack of precision in cloning of resistance genes or its identification in genomic clusters of source organism adds to the problem. More inputs are needed to supplement the high throughput functional genomics to enrich large experimental data of regulatory and structural genes (Kumar and Mysore 2011). This may certainly facilitate obtaining plentiful options of resistant genes for disease management in crop plants. The current advances in crop genomics, especially functional genomics and proteomics, will no doubt boost the development of disease resistance through transgenic crops.

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Chapter 18
Current Status of *Bacillus thuringiensis*: Insecticidal Crystal Proteins and Transgenic Crops

Devendra Jain, Vinod Saharan, and Sunil Pareek

Abstract *Bacillus thuringiensis* (Bt) is used to control agriculturally-important pests. It is a Gram positive spore-forming bacterium which produces parasporal proteinaceous inclusions during the sporulation phase. These crystalline parasporal inclusions are toxic to a wide spectrum of insects including the orders Lepidoptera, Coleopteran, Diptera, etc. The Bt insecticide proteins are toxic only after ingestion by the susceptible insects. The main steps involved when the Cry protein is ingested by the insect is comprised of solubilization of the protoxin, its enzymatic activation by terminal cleavage, receptor binding in brush border membrane of the midgut, pore formation, consequent disruption of ionic potential and destruction of the epithelial membrane leading to cell death. The first discovery of Bt was in 1901 when Ishiwata discovered a bacterium in Japan and in 1915, Berliner in Germany renamed it as *Bacillus thuringiensis*. Following a brief introduction, this chapter addresses the classification, the general structure of Cry toxin, its mode of action, strategies to improve the insecticidal activity of Cry proteins, transgenic plants developed using Bt genes, resistance to Bt toxins and resistance management, and an overall brief account of Bt and its insecticidal proteins, from 1901 to the present.

Keywords *Bacillus thuringiensis* • δ-endotoxins • Cry protein • Transgenic Bt plants • Insect resistance

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18.1 Introduction

Molecular biology and genetic engineering tools have provided plant scientists with unprecedented power to manipulate and develop novel crop genotypes towards a safe and sustainable agriculture in the twenty first century. Technologies and chemical inputs that have proven harmful to human health and the environment need to be replaced with safer alternatives to manage insect pests in agricultural ecosystems. Many insecticidal proteins and molecules are available in nature which are effective against agriculturally-important pests but are innocuous to mammals, beneficial insects and other organisms. Molecular tools can facilitate harnessing and deployment of these molecules in crop plants in a safe and sustainable fashion.

The insecticidal proteins produced by *Bacillus thuringiensis* (Bt) have provided a uniquely specific and effective tool for the control of a wide variety of insect pests. The multitude of insecticidal crystal proteins of Bt subspecies has spurred their use as a natural control agent with application in agriculture, forestry and human health. Trends in agriculture suggest that biological control will become increasingly important, particularly for integrated pest management (Bravo et al. 2011; Federici 1999; Romeis et al. 2006). Bt is a ubiquitous Gram positive, aerobic, endospore-forming bacterium which synthesizes crystalline proteins called *parasporal bodies* in the stationary phase of its growth cycle. The crystal is tightly packed by hydrophobic bonds and disulfide bridges; its most common shape is bipyramidal. These parasporal inclusions comprise either one or several related insecticidal proteins called δ-endotoxins which are insecticidal at low concentrations to the larvae of Lepidoptera, Coleoptera and Diptera (Hofte and Whiteley 1989). There are more recent reports of Bt isolates active against livestock ectoparasites (Gough et al. 2002), nematodes (Wei et al. 2003) and aquatic snails or molluscs (Salem et al. 2006). Scientific classification of Bt according to Berliner (1915) is as follows:

Kingdom: Eubacteria
Domain: Bacteria
Phylum: Firmicutes
Class: Bacilli
Order: Bacillales
Family: Bacillaceae
Genus: Bacillus
Species: *B. thuringiensis*

Bt was first discovered in 1901 by Ishiwata from diseased silkworms and named *Bacillus sotto*. Some years later, Berliner (1915) described Bt isolated from diseased Mediterranean flour moths (*Ephestia kuehniella*) obtained from Thueringen province in Germany. Although attempts were made in the early 1930s to commercialize Bt formulations, scientists were more preoccupied with the development of potent organic pesticides. By 1950, increased public awareness about the ill effects of pesticides on the environment led plant protectionists to opt for biological alternatives. The commercial use of Bt, as a suspension of spores and inclusions has been limited because of low persistence of toxins on plant surface due to inactiva-

tion by ultraviolet light, heat, pH and plant exudates. In spite of its environmentally-friendly reputation, Bt products have never represented a large share of the insecticide market, and are used primarily by organic farmers and gardeners and in forestry (de Maagd et al. 1999). This chapter addresses the status Bt and Bt toxin from its discovery in 1901. Mainly this chapter addresses ecology and the prevalence of Bt, Bt toxin structure, classification, mode of action, strategies to improve the insecticidal activity of Cry proteins, transgenic plants developed using Bt genes, insect resistance to Bt toxins and resistance management, effect of agronomical practices on Bt toxin production, applications of Bt beyond a potent insecticide and transgenic breeding.

18.2 Ecology, Prevalence and Isolation of Bt

18.2.1 Ecology and Prevalence of Bt

Since the discovery of Bt as an insect pathogen (Ishiwata 1901), it has long been believed that the organism occurs preferentially in the proximity of an insect-inhabiting environment. For the last two decades, many researchers have demonstrated that Bt is a common member of the micro flora of soil and aquatic habitats (Martin and Traverse 1989; Martinez and Caballero 2002; Uribe et al. 2003), phylloplane (Mizuki et al. 1999), freshwater (Ichimatsu et al. 2000), marine sediments (Maeda et al. 2000), free-living animals (Swiecicka et al. 2002), bank voles (Swiecicka and De Vos 2003), bird excreta in arid regions (Poopathi et al. 2014) as well as rice straw, grain dust, insect cadavers, compost, mammalian feces etc. (Meadows et al. 1992). This bacterium is widely distributed worldwide (Bernhard et al. 1997), ranging from the tropics to high elevations (Landen et al. 1994) and even Antarctica (Forsyth and Logan 2000).

18.2.2 Isolation of Bt

The distribution and presence of Bt mainly describes spores, because these are selectively obtained during isolation, which involves heating of the samples. Vegetative cells cannot be detected. Travers et al. (1987) standardized a procedure to isolate Bt from soil with a background of 10^9 bacteria per gram of soil, by the sodium acetate selection technique. This method allows the spores of unwanted bacterial species to germinate while preventing the desired bacteria from doing so. The unwanted bacteria, which enter the vegetative state, are eliminated by a controlled heat treatment. This procedure has been extensively used to isolate Bt from different ecological niches such as soil grain dust, rice straw compost and mammalian feces (Theunis et al. 1998). The isolation procedure was improvised by Johnson and Bishop (1996) following the penicillin cycling method, based on the observation

that all the strains in the culture collection were resistant to penicillin. Bt is also considered a part of the common leaf micro flora of many plants. Smith and Couche (1991) proposed three methods of collection by shaken flask, leaf lift and leaf scrub techniques to recover Bt from leaf surfaces. Widely used methodologies to isolate Bt from soil consist of a thermal shock treatment followed by selective germination of spores. Santana et al. (2008) documented that a preliminary 5 h dry-heat treatment largely enhanced the selectivity of Bt spores and enhances spore germination.

18.3 Classification of Bt and Its Crystal Proteins

Currently, over 50,000 Bt strains isolated from numerous screening procedures are distributed among various private and public collections throughout the world, and are considered to be potential reservoirs for novel toxins. Cry proteins are defined as a parasporal inclusion protein from Bt that exhibits toxic effects to a target organism, or any protein that has obvious sequence similarity to a known Cry protein (Crickmore et al. 1998). The diversity of Bt endotoxin gene and evidence of their possible multiplicity within an individual strain, makes tentative classification based on pathotype impossible. The first classification of Bt based on the flagellar antigens was developed by de Barjac and Bonnefoi (1962). A classification of Bt based on the H antigen was revised by de Barjac and Franchon (1990) and updated by Lecadet et al. (1999). Some 69 serotypes and 13 sub antigenic groups have now been identified, giving 82 serovar among the 3500 Bt isolates of the IEBC (International Entomopathogenic Bacillus Centre, France). The H serotype method provides a simple and efficient tool for classifying strains of Bt species. However, there are two problems: (a) strains lacking a parasporal inclusion, therefore considered to be *Bacillus cereus* and (b) autoagglutinated strains. As the number of Bt strains increases, the number of isolates falling into these two groups also increases.

Earlier classification of Bt proteins was according to both their host range and sequence homology (Hofte and Whiteley 1989). Bt toxins were grouped under four major classes based on their insecticidal spectrum. Lepidopteran-specific proteins were designated as CryI, Lepidopteran and Dipteran specific as CryII, Coleopteran-specific as CryIII and Diptera-specific are CryIV proteins. However, the nomenclature of Hofte and Whiteley (1989) failed to accommodate toxins belonging to the same class but with a different insecticidal spectrum. Hence, Crickmore et al. (1998) introduced a system of classification based on amino acid homology, where each protoxin acquired a name consisting of the mnemonic Cry (or Cyt) and four hierarchical ranks consisting of numbers, capital letters, lower case letters and numbers (e.g. Cry25Aa1). Thus, protein in less than 45 % homology differ in primary rank (Cry1, Cry2 etc.), and 78 % and 95 % identity constitute the border for secondary (Cry1A, Cry1B) and tertiary ranks (Cry1Aa, Cry1Ba), respectively. Quaternary rank was given to those proteins, which are more than 95 % similar in amino acid sequence (Cry1Aa1 and Cry1Aa2). The use of the quaternary rank (which distinguishes between toxins that are more than 95 % identical) is optional, only being used for the sake of clarity. It is to be noted that quaternary ranks are assigned to

each independently sequenced toxin gene; thus, despite the fact that some toxins have different quaternary ranks, they may in fact be identical. Additionally, other insecticidal proteins that are not related phylogenetically to the three-domain Cry family have been identified, among these, are binary-like toxins and Mtx-like toxins related to *Bacillus sphaericus* toxins, and parasporins produced by Bt (Crickmore et al. 1998). Details of Cry proteins nomenclature, holotypes, a full toxin list, Vip nomenclature, Parasporin nomenclature etc. are available at (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/).

18.4 Bt Crystal Morphology and Solubility

The crystal morphology in Bt is highly complex and exhibits different forms such as bipyramidal, cuboidal, spherical, squares and irregular (Chilcott and Wigley 1994) and insecticidal activities against different insect pests (Table 18.1). Bipyramidal crystals are active against Lepidopteran (Attathom et al. 1995); cuboidal crystals against Lepidopteran and Dipteran larvae or Lepidopteran larvae alone (Yammato and Mclanghlin 1981); spherical and irregular crystals are mostly mosquitocidal, often active against certain Coleopteran species (Krieg et al. 1983). Irregular crystals also include those with very little or no identified toxicity (Zelanzy et al. 1994). The crystal toxin is insoluble in water or inorganic solvents, but soluble in alkaline solvents. Cry1 proteins are soluble at pH 9.5, while the Cry2 proteins are soluble at a pH of about 12. Similarly, Cry4A, Cry5B and Cyt toxins are soluble at pH 9.5, while the Cry4D toxin requires a pH of 12. The Cry3A toxin, on the other hand, dissolves at pH below 4 and above 9.5 (Koller et al. 1992). The crystals can also be dissolved at neutral pH in the presence of detergents and denaturing agents like urea, β- mercaptoethanol, DTT and SDS.

Parasporin-2 is a protein toxin that is isolated from parasporal inclusions of Bt. Although Bt is generally known as a valuable source of insecticidal toxins, parasporin-2 is not insecticidal, but has a strong cytocidal activity in liver and colon cancer

Table 18.1 Insecticidal activity of Bt crystal proteins

Susceptible order	Cry proteins
Lepidoptera	Cry1A, Cry1B, Cry1C, Cry1E, Cry1F, Cry1I, Cry1J, Cry1K, Cry2A, Cry9A, Cry9B, Cry9C, Cry9I, Cry15A
Coleoptera	Cry1I, Cry3A, Cry3B, Cry3C, Cry7a, Cry8A, Cry8B, Cry8C, Cry14A, Cry23A
Diptera	Cry2A, Cry4A, Cry10A, Cry11A, Cry11B, Cry16A, Cry19A, Cry20A, Cry21A
Hymenoptera	Cry22A
Nematodes	Cry5A, Cry6A, Cry6B, Cry12A, Cry13A, Cry14A
Liverfluke	Cry5A

Source: Gatehouse et al. (2002)

cells. The 37-kDa inactive nascent protein is proteolytically cleaved to the 30-kDa active form that loses both the N-terminal and the C-terminal segments (Akiba et al. 2009).

18.5 Structure of Bt Toxin

The tertiary structure of crystal proteins Cry3Aa, Cry1Aa, Cyt2A, Cry2Aa, Cry3Bb, Cry4Aa, Cry4Ba, Cry8Ea1 etc. has already been described by X-ray crystallography and are available at a protein database (Fig. 18.1). All these structures display a very high degree of similarity and a conserved three-domain organization, suggesting a similar mode of action of the Cry proteins.

When the sequences of different Cry toxins are compared, five regions of homology are obvious. These are shown on the structure below (Fig. 18.2): Block 1 (orange); Block 2 (yellow); Block 3 (green); Block 4 (purple); Block 5 (magenta). Their distribution in the primary sequence is shown in Fig. 18.2.

The structure is composed of domains I, II and III. The N-terminal domain or domain I is composed of a bundle of 7 α-helices as shown in Fig. 18.1 (Cry1Ac) in which the central helix-α5 is hydrophobic and is encircled by 6 other amphipathic helices and this helical domain is responsible for membrane insertion and lytic pore formation. Mutations in domain I yield Bt toxins which bind to the receptor however, it does not produce lytic pore or fail to insert in the membrane. Domain II is believed to have a major role in receptor binding and thus in specificity determination. Domain II consists of three anti-parallel β-sheets with exposed loop regions which contain three unrelated carbohydrate binding proteins (including two lectins) which suggest that the exposed loops at the apex of this domain function as a lectin, which recognize carbohydrate determinants on the receptor (Boomserm et al. 2006). Domain III at the C-terminus of the molecule, is a sandwich of two twisted antiparallel beta-sheets. It was originally suggested that domain III may play some role in

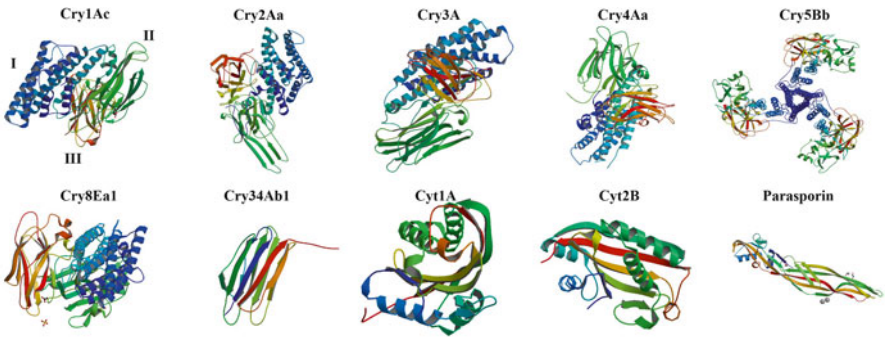

Fig. 18.1 3D structure of toxins produced by *Bacillus thuringiensis* (Source: Protein Data Base (http://www.rcsb.org/pdb/home/home.do))

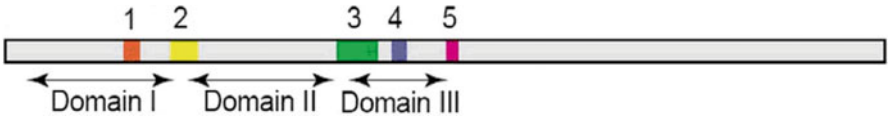

Fig. 18.2 Position of conserved regions in Cry protein sequence (Source: Adapted from de Maagd et al. 2003)

protecting the toxins against gut proteolysis. However, recent experiments involving reciprocal exchange of domain segments between toxins suggest that, domain III may also be a determinant of insect specificity/receptor binding (de Maagd et al. 1996). Cyt proteins, on the other hand, have a single α-β domain comprising of two outer layers of α-helix hairpins wrapped around a β-sheet (Li et al. 1996). Analysis of Cry protein sequences revealed that domains I and II have coevolved, where domain III sequences, revealed a different topology due to the swapping of domain III sequences among toxin (de Maagd et al. 2003). In the mature toxin, the N terminal domain (residues 1–272) is a pore forming seven helical bundles. The second domain (residues 273–473) is a receptor binding β prism. The third domain (residues 474–633) is implicated in determining both larval receptor binding (de Maagd et al. 1996) and pore-forming function (Schwartz et al. 1997).

18.6 Mode of Action of Bt Toxin in Lepidopteran Insects

The mode of action of δ-endotoxins is a multi-step process (Bravo et al. 2007). Bt insecticidal proteins are toxic to susceptible insects only after ingestion and processing in the midgut. The mode of action of Bt toxins can be understood by two hypothesis (Fig. 18.3); the first most widely-accepted hypothesis suggests that the primary action of Cry toxins is to lyse midgut epithelial cells in the target insect by forming pores in the apical microvilli membrane of the cells (Bravo et al. 2005). The second and more recent hypothesis suggests that toxicity could be related to G-protein mediated apoptosis followed by receptor binding (Zhang et al. 2006).

The main steps involved when the Cry protein is ingested by the insect are solubilization of the protoxin, its enzymatic activation by terminal cleavage, receptor binding in brush border membrane of the midgut, pore formation, consequent disruption of ionic potential and destruction of the epithelial membrane leading to cell death. The crystal inclusion bodies ingested by susceptible insect larvae dissolve these in the alkaline environment of the gut, and the solubilized inactive protoxins are cleaved by midgut proteases yielding 60–70 kDa protease-resistant proteins (Bravo et al. 2005). This alkaline pH is found in lepidopterans and dipterans, but in coleopterans the gut pH ranges from neutral to weakly acidic. Any toxins that are insoluble at this pH will not be toxic due to lack of their activation by gut proteases. Serine proteases are the main proteases in lepidoptera and diptera, whereas cysteine

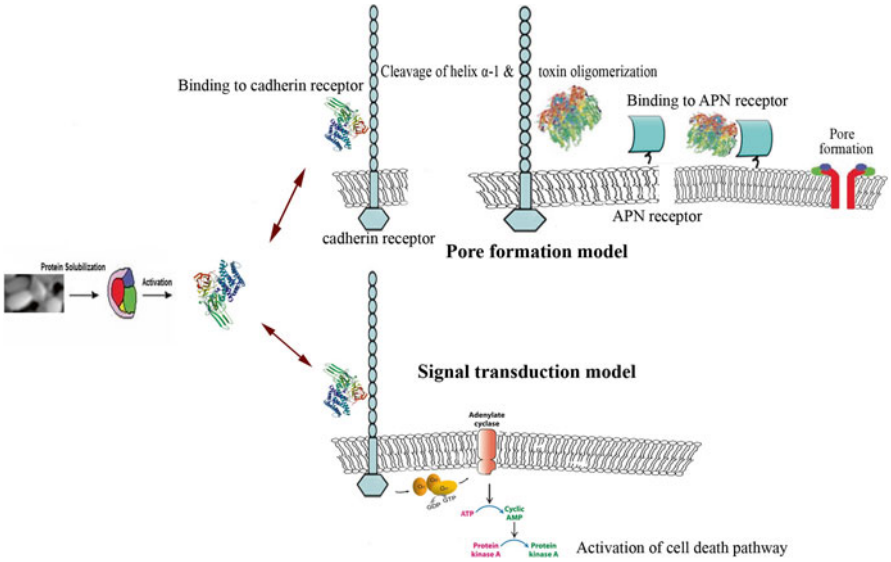

Fig. 18.3 Mode of action of Bt toxins (Source: Adapted from Bravo et al. 2007)

and aspartic proteases are the main proteases in coleoptera. Toxin activation involves the proteolytic removal of an N-terminal peptide (25–30 amino acids for Cry1 toxins, 58 residues for Cry3A and 49 for Cry2Aa) and approximately half of the remaining protein from the C-terminus in the case of the long Cry protoxins. The activated toxin then binds to specific receptors on the brush border membrane of the midgut epithelium columnar cells before inserting into the membrane. For Cry1A toxins, the main receptor proteins are cadherin-like protein (CADR), glycosylphosphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN), GPI-anchored alkaline phosphatase (ALP) and a 270 kDa glycoconjugate (Jurat-Fuentes et al. 2004; Valaitis et al. 2001). The activated Cry toxin is bound to a cadherin receptor located in the microvilli of the midgut cells. The pore-formation model proposes that interaction with activated toxin and cadherin facilitates further proteolytic cleavage and induces the cleavage of helix α1 and triggers toxin oligomerization. These toxin oligomer then bind to another receptor, which is glycosylphosphatidylinositol (GPI) anchored aminopeptidase N (APN) in *Manduca sexta* or alkaline phosphatase in *Heliothis virescens*. In a final step, the toxin oligomer inserts into lipid raft membranes and forms lytic pores and subsequently causes cells to burst, resulting in the death of the larva.

In another hypothesis, based on the signal transduction model, the binding of Cry1A to the cadherin receptor is assumed to trigger a cascade pathway involving the stimulation of a G protein and adenylate cyclase which increases cAMP production, resulting in the activation of protein kinase A; activation of the adenylate cyclase/protein kinase A signaling pathway initiates a series of cytological events

that include membrane blebbing, appearance of nuclear ghosts and cell swelling followed by cell lysis (oncotic cell death). The findings from most of the research to date supports the pore-formation model; however, the signal transduction model was proposed based on research performed on insect cell lines.

18.7 Mode of Action of Cry and Cyt Toxins in Mosquitos

Bt ssp. *israelensis* (Bti) is highly toxic to mosquito species, and produces crystal inclusions composed of Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa and Cyt2Ba toxins (Berry et al. 2002). In mosquitos the crystals ingested by susceptible larvae dissolve in the alkaline gut environment, releasing soluble proteins. In the Cry type mosquitocidal protoxins, gut proteases removes N-terminal and C-terminal ends and also cleaves intramolecularly which results in two fragments, and these two fragments remain associated and impart toxicity (Yamagiwa et al. 2004). In the case of Cyt toxins, which are also synthesized as protoxins, and small portions of the N-terminus and C-terminus, are removed to activate the toxin, resulting in a monomeric protein with hemolytic activity. It is proposed that Cry toxins bind to specific protein receptors (GPI-ALP or GPI-APN) in the microvilli of the mosquito midgut cells, whereas in contrast, Cyt toxins do not bind to protein receptors but directly interact with membrane lipids inserting into the membrane and forming pores (Gill et al. 1987; Li et al. 1996; Promdonkoy and Ellar 2003; Thomas and Ellar 1983) or destroying the membrane by a detergent-like interaction (Butko 2003).

18.8 Insecticidal Proteins of Bt

Bt produces an array of toxins such as β- exotoxin, proteases, phospholipases, vegetative insecticidal proteins (VIPs) and ICP (cry and cyt), which are toxic to insects. Among all the above mentioned toxin chitinases, VIP and ICP (cry and cyt) are promising candidates for insect control.

18.8.1 Cloning and Expression of Chitinase Gene of Bt

Chitin, the β-(1, 4) linked homopolysaccharide of N-acetylglucosamine, is the second most abundant polysaccharide in nature. It occurs in insects as a major component of the cuticle and of the peritrophic membrane, a protective sleeve lining the gut of many insects (Cabib 1987). Few Bt strains produce and excrete chitinase into the culture media. Chitinases have been successfully used in combination with Bt delta-endotoxins forming crystals in order to enhance their insecticidal activities (Driss et al. 2011; Sirichotpakorn et al. 2001). The chitinase genes from different

strains of Bt e.g. *pakistani*, *kenyae*, serovar *alesti*, serovar *sotto* and HD1 have been cloned and properties of the encoded protein have been discussed in detail (Barboza-Corona et al. 2003; Lin and Xiong 2004; Thamthiankul et al. 2001; Zhong et al. 2005).

18.8.2 Cloning and Expression of the Vip Gene of Bt

A novel class of protein called vegetative insecticidal protein (Vip), produced by Bt during its vegetative stages of growth, has been identified (Estruch et al. 1996). Unlike Cry proteins, Vips are about 88.6 kDa in size, not parasporal, having a putative bacillar secretory signal at the N-terminal which is not processed during its secretion. It does not show any homology with the known crystalline insecticidal proteins. This structural dissimilarity is indicative of a possible divergent insecticidal mechanism from the other known Bt toxins. The symptoms produced by Vips are similar to those caused by Cry proteins, but it develops 48–72 h after ingestion, whereas in the latter it takes only 16–24 h (Yu et al. 1997). The Vip3A shows activity against a wide variety of lepidopteran insect pests, including *Agrotis ipsilon*, *Spodoptera frugiperda*, *S. exigua* and *Heliothis zea* (Estruch et al. 1996; Lee et al. 2006). The Vip3A gene of Bt was cloned and characterized by Doss et al. (2002) which showed high toxicity against *Bombyx mori* and mosquito (*Culex quinquefasciatus*). The Vip1A gene encodes 100 kDa protein and yields 80 kDa active protein after processing. The 80 kDa Vip1A protein is reported to be toxic to western corn root worm larvae in conjunction with the Vip2A protein, whose coding region is located immediately upstream (Warren et al. 1994). Hernandez-Rodriguez et al. (2009) for the first time screened all vip1, vip2 and vip3 genes from the 507 Bt strains. In India, Bhalla et al. (2005) screened the vip genes from local isolates and cloned and expressed a novel vip3Aa14 gene. Individually vip has been successfully expressed in monocots and dicot plants and efforts to pyramid vip in the Bt transgenic crops are under way in several laboratories.

18.8.3 Cloning and Expression of Bt Cry Genes

Cloning of the first crystal protein gene (cry) of Bt was reported by Schnepf and Whiteley (1981). As of 24-08-2014, 800 different Bt toxins had reportedly been cloned and 305 Bt holotype toxins, all available at (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). Cloning of cry genes provides an opportunity to express the cloned gene in acrystalliferous Bt or *Escherichia coli* to ascertain the insecticidal activities of their proteins. The reintroduction of cloned genes into Bt also provides a system to study factors regulating the expression of delta endotoxin genes. Using such a system, it has been discovered that two endotoxin genes, cytA and cry2Aa, require an accessory protein to be co-expressed in order that their

products may form crystalline inclusions (Adams et al. 1989; Crickmore and Ellar 1992). Bt strains isolated from different agroclimatic regions of India were found to harbor cry family genes (Prabagaran et al. 2002; Ramalakshmi and Udayasuriyan 2010).

The polymerase chain reaction (PCR) is a molecular tool widely used to characterize the insecticidal bacterium Bt strain collections (Baron et al. 2005; Ben-Dov et al. 1997; Bravo et al. 1998; Jain et al. 2006, 2012; Vidal-Quist et al. 2009). This technique is a highly sensitive method of rapidly detecting and identifying target DNA sequences and requires minute amounts of DNA and allows screening of many Bt samples to classify them and predict their insecticidal activities. The PCR based identification of Bt cry genes was first developed by Carozzi et al. (1991) and they designed primers from cry1Ab, cry3A and cry4A genes, for identification of Lepidoptera, Coleoptera and Diptera active strains, respectively. The efficacy of PCR in identifying the large family of cry genes, with amino acid identities ranging from less than 45 % to more than 95 %, is based on the presence of conserved regions. For practical reasons, primer pairs designed from highly-conserved regions and recognizing entire cry gene sub families are often used in a preliminary screening prior to performing a second PCR with specific primers. Another strategy for the screening is based on the multiplex PCR which uses more than two primers in a mixture of the same reaction (Juarez Perez et al. 1997). Usually, a single universal primer is combined with several specific oligonucleotides that recognize individual genes. The PCR-restriction fragment length polymorphism (RFLP) typing system is a facile method to detect both known and novel cry genes existing in Bt strains (Kuo and Chak 1996). One novel cry1I type from Bt was found and characterized by this method (Song et al. 2003). Beron et al. (2005) reported a two-step based approach which allows amplify cation of currently known as well as novel cry gene sequences using degenerate primers and characterization of amplicons by sequencing.

18.9 Molecular Biology of Toxins

18.9.1 Localization and Molecular Organization of Toxin Genes

Most Bt strains harbor an array of plasmids with varying size of 1.4–180 MDa. Hybridization experiments indicate that the plasmids from various strains fall into two size groups: small plasmids (<10 MDa, high copy number) with some degree of relatedness between them and no known function, and large plasmids (>30 MDa, low copy number) sharing homologous DNA sequences (Lereclus et al. 1982). The larger and low copy number plasmids are very stably maintained in the cell and they replicate by the theta (θ) mechanism. Evidence for a correlation between crystal protein synthesis and the presence of a particular plasmid was provided by the curing experiments leading to the loss of large plasmid with concomitant loss of crystal protein

synthesis (Gonzalez et al. 1981). The results indicated that toxin genes are found on the large plasmids (40–150 MDa) of many strains of Bt, but in a few strains (e.g. Bt ssp. *entomocidus*, Bt ssp. *aizawai*) they are localized on the chromosome (Sanchis et al. 1988; Sekar et al. 1987). Many Bt strains harbor more than one cry gene.

18.9.2 Regulation of Insecticidal Crystal Protein Production

The production of most Cry proteins in Bt starts at the stationary phase and accumulates throughout the sporulation, resulting in the appearance of the parasporal inclusion within the mother cell. The Cry proteins generally account for up to 25 % of the dry weight of the sporulated cells. The amount of crystal protein produced by the Bt culture in the laboratory and the size of the crystals (Du et al. 1994) indicate that each cell has to synthesize 10^6 to 2×10^6 δ – endotoxin molecules during the stationary phase to form a crystal. This is a massive production of protein and presumably occupies a large proportion of cell machinery, which is discussed in detail by Deng et al. (2014). The high level of crystal protein synthesis in Bt and its co-ordination with the stationary phase are controlled by a variety of mechanisms occurring at the transcriptional, post transcriptional and post translational levels. In Bt, most of the cry genes are expressed only during sporulation; few genes are expressed during the vegetative phase (Moran 1993).

18.9.3 Sporulation Dependent Cry Gene Expression

Production of a large amount of insecticidal crystal proteins encoded on large plasmids is largely dependent upon the mother cell, Bt specific transcription systems attributable to sporulation. The cry1A gene is a typical example of sporulation dependent cry gene that is only expressed in the mother cell compartment of Bt. Two transcription start sites (BtI and BtII) have been mapped in cry1A transcripts, defining two overlapping, sequentially activated promoters (Wong et al. 1983). BtI is active between T_2 and T_6 of sporulation and BtII is active from T_5 onwards. Transcription from Bt I promoter is initiated by a form of RNA polymerase containing an alternative sigma factor σ^{35} (Brown and Whiteley 1990). From the results of in vitro transcription experiments it appears that at least three cry genes (cry1B, cry2A and cytA) contain either BtI alone or both BtI and BtII. Many other cry genes (e.g. cryIVA, cryIVB and cryIVD genes from subsp. *israelensis* and cry34 and cry40 genes from *thompsoni*) are considered to be sporulation-stage specific gene because their promoter region contains consensus sequences (Dervyn et al. 1995; Yoshisue et al. 1993). In the middle stages of sporulation, cry4A is most actively transcribed from the promoter cry4A-P1. The proximal transcriptional starting point of cry4A, which is under the control of the promoter P1, is used in *Bacillus subtilis* in the middle stage of sporulation (Komano et al. 2000).

18.9.4 Sporulation Independent Cry Gene Expression

The cry3A gene, isolated from the coleopteran active Bt var. *tenebrionis* (Sekar et al. 1987), is a typical example of a non- sporulation-dependent cry gene. It has been shown that the cry3Aa promoter is weak, but significantly expressed during the vegetative phase of growth (unlike the cry1A promoter), is activated at the end of the exponential growth, and remains active only until about T_8 in sporulation medium (to indicate the onset of stationary phase and T_n is the number of hours after time zero). Unlike BtI and BtII, the cry3A promoter is similar to the promoter recognized by the primary sigma factor of vegetative cells, σ^A (Agaisse and Lereclus 1994a). The sporulation of cry3A is not dependent on sporulation-specific sigma factors either in *Bacillus subtilis* (Agaisse and Lereclus 1994b) or in Bt (Salamitou et al. 1996). The cry3A promoter, although located unusually far upstream of the start codon (position-558), resembles a promoter recognized by the primary sigma factor (σ^A) of vegetative cells. Moreover, cry3A expression is increased and prolonged in mutant strains which are unable to initiate sporulation (Malavar and Baum 1994). The genes involved in cry3A regulation have not been identified.

18.10 Expression of Cry Genes in Other Microorganisms

Low persistence of Bt pesticides due to inactivation by ultraviolet light, heat, leaf exudates and pH, can be improved by transfer of cry genes to other better-persisting microbes. To combat this, scientists opted for DNA manipulations and expressed the cloned cry genes in other microorganisms preferably plant associating microbes (Table 18.2). The first report of such an expression was established, when Monsanto scientists successfully expressed a cry1Ab gene into a root-colonizing bacterium *Pseudomonas fluorescens* (Watrud et al. 1985). It conferred better control of cabbage and potato from lepidopteran and coleopteran pests, respectively, due to an approximately two-fold increase in foliar persistence (Carlton 1996). Interestingly, Ruan et al. (2002) expressed the mcl gene from *P. maltophilia* in Bt, which significantly protects the Bt toxin from UV degradation, as melanin has the ability to act as UV absorber, a recombinant Bt strain producing melanin provides stability for Bt preparation.

18.11 Strategies to Improve the Insecticidal Activity of Cry Proteins

Bt Cry toxins have been widely used in the control of insect pests either as spray products or expressed in transgenic crops. Cry toxins are specific against susceptible larvae and although they are often highly effective, some insect pests are not

Table 18.2 Cloning and expression of Bt gene in heterologous hosts

S.N.	Bt protein	Microbial host	References
1.	Cry1AC	*Pseudomonas cepacia*	Stock et al. (1990)
2.	Cry4B	*Caulobacter crescentus*	Thanabalu et al. (1992)
3.	Cry4D	*Agmenellum quadruplicatum* PR-6	Murphy and Stevens Jr. (1992)
4.	Cry1Aa	*Bacillus megaterium*	Bora et al. (1994)
			Sudarsan (1994)
5.	Cry1Aa	*Azospirillum*	Udayasuriyan et al. 1995
6.	Cry1AC	*Clavibacter xyli*	Lampel et al. (1994)
7.	Cry2A	*B. cereus*	Moar et al. (1994)
8.	Cry4A,	*Anabaena* sp. Stain PCC 7120	XiaoQiang et al. (1997)
	Cry 11A		
9.	Cry1Ac	*P. fluorescens*	Herrera et al. (1997)
10.	Cry11A	*B. sphaericus*	Poncet et al. (1997)
11.	Cry1Ac	*Azospirillum*	da Costa Lima et al. (2000)
12.	Cry1Ac	*B. polymixa*	Sudha et al. (1999)
13.	Cry3A	*Gluconoacetobacter diazotrophicus*	Salles et al. (2000)
14.	Cyt2Aa1	*Pichia pastoris*	Gurkam and Ellar (2003)
15.	Cry1Ab	*B. & B. licheniformis*	Theoduloz et al. (2003)
16.	Cry1Ac	*Baculovirus*	Chang et al. (2003)
17.	Vip3	*Photorhabdus temperata*	Jamoussi et al. (2009)
18.	Cry11A	*B. brevis*	Roh et al. (2010)

affected by them or show low susceptibility. In addition, the development of resistance threatens their effectiveness, so strategies to cope with all these problems are necessary (Padro-Lopez et al. 2009). The following different strategies can be used to improve insecticidal activity of Cry toxins.

18.11.1 Potentiation of Cry Toxin Activity by Additional Proteins

Serine Protease Inhibitors Several serine protease inhibitors increase the insecticidal activity of Cry toxins up to 20-fold at extremely low-level concentrations (Maclntosh et al. 1990). Genetically modified tobacco plants expressing a protease inhibitor (*Curcurbita maxima* trypsin protease inhibitor) fused to a truncated Cry1Ab toxin showed a six-fold increase in specific insecticidal activity against tobacco budworm when compared with plants expressing only Cry toxin protein (Maclntosh et al. 1990).

Chitinase Ding et al. (2008) reported that increasing the levels of endo chitinases in the larval midgut or the addition of external chitinases to Cry toxin preparations

increases their efficacy and potency up to ten-fold. It was proposed that chitinases elevate the larvicidal effect by perforating the peritrophic membrane, increasing the accessibility of the Cry toxin to the epithelial membrane where receptors are located.

Cyt Toxins Cyt proteins are hemolytic and cytolytic toxins produced by some Bt strains. Perez et al. (2005) reported that Cyt1Aa synergizes the Cry11Aa toxic activity by functioning as a membrane-bound receptor of Cry11Aa. Perez et al. (2007) proposed the mechanism that Cyt1Aa inserts into the midgut epithelium and exposes protein regions that are recognized by Cry11Aa, thereby facilitating the oligomerization of Cry11Aa and its pore-forming activity.

Peptide from Cadherin Receptor CR12-MPED Cadherin-like receptors are transmembrane proteins with a cytoplasmic domain and an extracellular ectodomain with several cadherin repeats (CADR) (12 in the case of the cadherin receptor of *Manduca sexta*) (Vadlamudi et al. 1995). Chan et al. (2007) reported that the CADR12 binding site (12th cadherin repeat or CR12) has an important role in binding to Cry toxins and enhancement of the toxicity. CR12 fragment also binds to the apical membrane of larvae gut cells with high affinity suggesting that this will increase the number of binding sites in the microvilli membrane of the insect and hence will increase the toxicity.

18.11.2 Modifications in the Cry Toxin Gene

Enhanced toxicity of Cry proteins by protein engineering requires complete elucidation of structure and assignment of physiological function to different structural domains. Protein engineering not only reveals the mechanism by which δ-endotoxins work, but it can generate toxins with enhanced toxicity with or without new brush border membrane vesicles (BBMV) binding properties. These toxins could be used in resistance management as alternatives for the toxins already in use to which insects may become resistant by losing receptors. About 40 % of the currently identified Bt toxins are not active on insects, due to various reasons like low solubility in the insect gut environment, lack of binding to BBMV in the larval midgut, presence of protease cleavage sites. Knowledge of δ-endotoxins can be utilized to make these inactive toxins active by protein engineering.

Based on the huge information on amino acids sequence and three dimensional structure of Cry proteins, several attempts have been made to engineer the Cry proteins to fulfill various objectives such as to: (a) enhance the toxicity of Cry proteins (b) widen the host spectrum of Cry proteins (c) manage the resistance development in insects and (d) analyse the structural functional relationship of each domains. Several molecular biology tools have been utilized to engineer Cry proteins such as: (a) site directed and random mutagenesis (b) in vivo homologous recombination and domain swapping (c) DNA shuffling (d) introduction of specific proteolytic cleavage sites and (e) deletion of a small region of the toxin.

Site Directed and Random Mutagenesis Some of the classical examples of protein engineering via site directed and random mutagenesis are as follows:

- A mutation ($H_{168}R$) in the helix α5 of cry1Ac domain I caused a two-fold increase in toxicity against *Manduca sexta* (Wu and Aronson 1992).
- In cry1Ab, a combination of mutation in the α8 loop and loop2 resulted in a 32-fold increase in toxicity to *Lymantria dispar* over the background gene product and a four-fold improvement over the previously best known gene product (cry1Aa) (Rajamohan et al. 1996a, b).
- Abdullah et al. (2003) reported that few amino acid residue changes in domain II loop 3 of Cry4Ba can introduce an increase in toxicity towards the mosquito genus.
- Wu et al. (2000) reported that two mutants in this loop, named A1 and A2, containing multiple mutations each (A1: R345A, Y350F, Y351F; and A2: R345A and the deletion of DY350Y351), showed 3- and 11-fold higher toxicity against the coleopteran *Tenebrio molitor*, respectively, and showed increased binding affinity. There are also mutations in loop 3 of Cry3A toxin that also showed a moderate increase in toxicity (2.4-fold) to *T. molitor* larvae.

Domain Swapping and In Vivo Homologous Recombination Some of the classical examples of protein engineering via domain swapping and in vivo homologous recombination are as follows:

- Ge et al. (1991) reported 30-fold enhanced toxicity of Cry1Aa protein towards the *Heliothis virescens*, when Cry1Aa amino acid residues (450–612) were replaced by Cry1Ac amino acids.
- Bosch et al. (1994) reported that transfer of domain III of Cry1C to Cry1E resulted in a recombinant toxin which is toxic to the *Spodoptera exigua* and *Mamastra brassicae* whereas parental toxin (Cry1E) was not toxic against *S. exigua* and *M. brassicae*. Therefore domain swapping would be a valuable tool to manage resistance development.
- Naimov et al. (2001) reported a hybrid Cry1 protein (Cry1Ba/Cry1Ia) with increased toxicity against coleopteran insects.
- de Maagd et al. (2000) reported that several Cry1 toxins with low or no specificity against *S. exigua* including Cry1Ab, Cry1Ac, Cry1Ba and Cry1Ea, become active when their domain III is replaced by that of Cry1Ca.
- Caramori et al. (1991) reported that replacement of residues 450–612 (domain III) of Cry1Aa by those of Cry1Ac resulted in a 300-fold increase in toxicity against *H. virescens*.

DNA Shuffling DNA shuffling is a powerful process for directed evolution, which generates diversity by recombination. DNA family shuffling mimics and extends classical breeding methods by recombining more than two parental genes, or genes from different species, in a single DNA shuffling reaction. In this method, the gene is subjected to random mutations and is then screened for improved ones. In this method, the acquisition of genes encoding improved proteins is done in two steps. In the first step, a single is mutagenized, and desired mutant genes are selected. In the second step, the mutant genes are fragmented by DNase I and the purified frag-

ments are extended by repeated cycles of overlap extension into full length genes that contain a novel combination of the parental mutations. Point mutations are also introduced during the shuffling process (Stemmer 1994). With the family shuffling using ssDNA, chimeric genes were obtained at a rate of 14 % which was much higher than the rate of 1 % obtained by shuffling with dsDNA. With the method using restriction enzyme cleaved DNA fragments, all shuffled genes obtained were actually chimeric and divergent. Lassner and Bedbrook (2001) described that when the Cry3 gene was subjected to DNA shuffling not only toxicity of Cry3 protein was improved (3.8-fold) but it was also toxic to lepidopteran insects. Craveiro et al. (2010) reported that modified Cry1Ia toxins generated by DNA shuffling as having increased activity against sugarcane giant borer. DNA shuffling coupled with the phage-display technique has been valuable for the generation of genetic diversity and for selection of variants showing binding affinity to specific protein targets.

Introduction of Specific Proteolytic Cleavage Sites Walters et al. (2008) proposed that enhanced cleavage of this toxin at this proteolytic site permitted the subsequent binding of the activated toxin to the receptors present in the midgut cells. The Cry3A toxin, which possess low toxicity against *Diabrotica virgifera* (an important pest of maize), the toxicity have been increased by three-fold after introduction of a chymotrypsin/cathepsin G site in the loop between helix α-3 and helix α-4.

Deletion of Small Regions of the Toxin As per the most recent model of toxin action, binding of Cry toxins to the cadherin receptor is essential for removal of the helix α-1 and this cleavage then promotes oligomerization of the toxin to form a pre-pore structure. Mutations in midgut cadherin that bind Cry1Ac are linked with insect resistance and represent the most common mechanism of resistance (Xu et al. 2005). Deletion in the amino-terminal region including helix α-1 of Cry1A toxins resulted in Cry toxins that form oligomers in the absence of cadherin receptor. Such modified toxins killed insects that had developed resistance to Cry1A toxins caused by mutations in the cadherin gene. The modified toxins were also effective against insects which had acquired reduced susceptibility to native Bt toxins due to diminished expression of cadherin protein by cadherin gene silencing with RNA interference (Soberon et al. 2007). Deletions of small fragments at their amino termini resulted either in increased toxicity or in toxins that could be useful for countering insect resistance to native Cry1A toxins either applied as spray products or expressed in transgenic crops. Such altered toxins may be particularly effective in insects that became resistant due to changes in the first toxin receptor.

18.12 Application of Bt in Agriculture: Bt Spray Formulations

The Cry proteins studied thus far have been verified to be non-pathogenic to mammals, birds, reptiles and amphibians. These toxins are highly specific and lethal to insects and invertebrate pests. Individual toxins have narrow specificity, but the vast array of toxins reported in the literature can cover a very broad spectrum of insect

pests. To fully realize the potential of Bt δ-endotoxins as biopesticides, progress is required in the following areas.

(a) Increase the yield or efficiency of toxin protein production.
(b) Gain a satisfactory understanding of the mechanism of toxicity to allow engineering of the toxins for maximum activity.
(c) Continue to isolate new strains with novel toxin structures and activities.

Bt formulations could be applied in solid (powder or granules) or liquid forms. Presently over 400 of Bt-based formulations are registered for commercial application (Ahmedani et al. 2008). These Bt formulations can be applied directly in the form of field sprays (Ali et al. 2010). The increased popularity of Bt formulations over synthetic chemicals is mainly because of the non-selective lethal effect of the chemical pesticides and the rapid development of resistance to these synthetic insecticides. Able, Biodit, Cutlass, Dipal, Foray, Javalin, Thuricide, Vectobac etc. are some of the products that contain Bt spores and crystal mixture that are commercialized and registered for agriculture use (Table 18.3) (Whalon and Wingerd 2003).

18.13 Application of Bt in Agriculture: Transgenic Approach

The delivery of Bt insecticidal crystal proteins through spray formulations, engineered Bt and other bacteria has certain limitations. The biopesticidal sprays suffer from short half-life, physical removal (wind and rain) and inability to reach burrowing insects. Engineered bacteria very often proliferate at a rate and quantity not sufficient to kill the target insect pest. These disadvantages can be overcome if the Bt Cry proteins are expressed in the plant cells at levels sufficient enough to kill the larvae. Some of the advantages of transgenic Bt plants are listed as follows.

(a) Environmental benefits from the absence of pesticide drift and absence of residual pesticides.
(b) Absence of effects on non-target species.
(c) Root pests, stem and fruit borers (hidden pests) that are not affected by conventional Bt sprays are killed by toxin-producing Bt plants.
(d) Reduced exposure of farmers, farm labor and non-target organisms to the pesticides.
(e) Increased activity of *natural enemies* because of reduction in pesticide sprays (Sharma and Ortiz 2002).

Thus Bt crops promise savings on insecticidal usage and enable a greater involvement of other biocontrol strategies in IPM by reducing reliance on broad-spectrum pesticides. The first reported use of the δ endotoxin gene expressed in plants for insect control occurred in 1987 (Barton et al. 1987; Vaeck et al. 1987). Vaeck et al. (1987) produced the transgenic tobacco expressing full length cry1Ab gene from Bt var. *berliner* 1715. Transgenic tobacco plants were obtained by leaf disk transformation of *Nicotiana tabacum* var. Petit Havana SR1. The constructs tested in

Table 18.3 Natural and genetically modified Bt products registered for agricultural use

Bt strain	Company	Product	Target insect
Natural Bt stains			
kurstaki HD-1	Abbot, USA	Biobit, Dipel, Foray	Lepidoptera
kurstaki HD-1	Thermo Trilogy, USA	Javelin, Steward, Thuricide	Lepidoptera
Kurstaki	Abbot	Bactospeine, Futura	Lepidoptera
Kurstaki	Thermo Trilogy	Able, Costar	Lepidoptera
Aizawai	Abbot	Florbac, Xentari	Lepidoptera
Tenebrionis	Abbot	Novodar	Coleoptera
Tenebrionis	Thermal Trilogy	Trident	Coleoptera
Kurstaki	Bio Dalia, Israel	Bio-Ti	Lepidoptera
Kurstaki	Rimi, Israel	Bitayon (granular feeding baits)	*Batrachedra amydraula*
Galleriae	Tuticorin Alkali chemicals & fertilizers, India	Spicturin	Lepidoptera
YB-1520	Huazhong Agric. University, China	Mainfeng pesticide	Lepidoptera
CT-43	Huazhong Agric. University, China	Shuangdu	Lepidoptera Coleoptera
			Diptera
Genetically modified Bt Stains			
aizawai recipient *kurstaki* donor	Thermo Trilogy	Agree, Design (transconjugant)	Lepidoptera
kurstaki recipient	Ecogen, USA	Condor, Cutlass transconjugant), CRYMAX, Leptino	Lepidoptera
Kurstaki	Ecogen	Leptinox (recombinant)	Lepidoptera
kurstaki recipient	Ecogen	Raven (recombinant)	Lepidoptera
δ endotoxin encapsulated in *Pseudomonas fluorescens*	Mycogen, USA	MVP	Lepidoptera Coleptera
		MATTCH	
		MTRACK	
		CellCap	

Source: Navon (2000)

tobacco included full length genes, a fragment of cry1Ab gene and two constructs with translational fusion between the amino terminal fragment of cry1Ab and a neo (Neomycin Phosphotransferase) gene that conferred kanamycin resistance to plants. Tobacco transformed with truncated genes and δ-endotoxin- neo fusion gene constructs produced 75–100 % mortality against *Manduca sexta*. The plants transformed with full-length endotoxin genes produced very little delta-endotoxin protein and were non-insecticidal.

Adang et al. (1987) generated transgenic tobacco containing the full length cry1Ac gene from Bt var. *kurstaki* HD-73 using a binary vector system and

Agrobacterium tumefaciens. Barton et al. (1987) studied the expression of full length and NH_2 terminal fragment of cry1Aa gene from Bt var. *kurstaki* HD-1 in transgenic tobacco *Nicotiana tabacum* cv. Havana 425. At least one plant with a truncated gene showed low levels of Cry1Ac toxin (2 μg/mg soluble proteins) and some degree of toxicity towards tobacco horn worm was identified. None of the plants with the full-length gene produced detectable levels of cry1Aa mRNA or insecticidal activity. Truncated versions of the cry1Ab gene were used for transformation of tomato (Fischhoff et al. 1987).

The results of field tests of first generation transgenic tobacco concluded that the expression levels of Bt genes in plants were relatively low due to the truncated transcripts. To enhance Bt δ endotoxin gene expression, the genes with sequence modification or new tissue specific promoters were introduced. Perlak et al. (1991) examined several versions of the modified cry1Ab and cry1Ac genes in both transgenic tobacco and tomato to determine more closely the increased expression associated with various sequence modifications. Koziel et al. (1993) synthesized a truncated cry1Ab gene redesigned to replace the bacterial codons with maize preferred codons and with 65 % G+C content when compared with 37 % for the native gene. Different versions of cry genes effective against different orders of insects have been identified and introduced into crop plants (Table 18.4).

The synthetic cry1A genes were introduced into different crops such as rice (Alam et al. 1999; Cheng et al. 1998; Datta et al. 1998), maize (Douville et al. 2009; Koziel et al. 1993), cotton (Ibargutxi et al. 2006; Perlak et al. 1990; Van Wyk et al. 2009), potato (Kumar et al. 2010), eggplant (Kumar et al. 1998), tomato (Mandaokar et al. 2000), cabbage (Bhattacharya et al. 2002), cauliflower (Chakrabarty et al. 2002), castor bean (Malathi et al. 2006), pine (Barraclough et al. 2009). Shu et al. (2000) reported the development of transgenic rice plants with a synthetic cry1Ab gene and high-level resistance to eight lepidopteran rice pest species. Transgenic crop plants with synthetic cry3A against Colorado potato beetle (Adang et al. 1993) and cry9A gene for protection against *Plutella xylostella* (Kuvshinov et al. 2001) have been reported. Two new δ endotoxins from Bt having molecular masses of 14 kDa and 44 kDa were produced in maize engineered with the corresponding genes for protection from corn root worms (Moellenbeck et al. 2001).

The first field trials with transgenic crops expressing the Bt toxins were conducted in 1986 with tobacco. In 1995, the first transgenic plants, maize expressing the Cry1Ab toxin (Maximiser™ from Novartis), cotton expressing the Cry1Ac toxin (Bollgard™ from Monsanto) and potato expressing Cry3A toxin (Newleaf™ from Monsanto) were approved for sale in the USA (Jouanin et al. 1998). Syngenta is currently developing VipCot™, transgenic insect-resistant cotton that expresses both Vip3A and Cry1Ab toxins (Kurtz et al. 2007). VipCot employs vegetative insecticidal protein discovered in 1994 by Syngenta, Vip is structurally and functionally different from the endotoxins employed in current traits.

The global hectarage of biotech crops has increased more than 100-fold from 1.7 million ha to over 175 million ha in the last 18 years, out of which ~25 million ha are Bt crops, which makes biotech crops the fastest adopted crop technology among farmers from ~30 countries. In 2013, a record 18 million farmers (of which over

Table 18.4 List of Bt transgenic crops

Crop	Bt gene used	Against the pest	References
Maize	cry1Ab	*Heliothis zea*	Koziel et al. (1993)
	cry1Ac	*Pectinophora gossypiella*	Buschman et al. (1998)
Potato	cry3A	*Leptinotarsa decemlineata*	Perlak et al. (1993)
	cry1Ab	Potato tuber worm	Duck and Evola (1997) and Kumar et al. (2010)
	cry1Ac	Potato tuber worm	Ebora et al. (1994)
	cry5	Potato tuber worm	Douches et al. (2004)
Cotton	cry1Ac	Cotton ball worm	Perlak et al. (1990) and Jenkins et al. (1997)
	cry1Ab	Pink ball worm	Wilson et al. (1992)
	vip3Aa	Lepidopteran insects	Artim (2003)
	cry1Ac, cry2Ab	Lepidopteran insects	Héma et al. (2009)
Tomato	cry1Ab	Tobacco hornworm	Delannay et al. (1989)
Brinjal	cry1Ab	Fruit borer	Kumar et al. (1998)
	cry3B	Fruit borer	Iannacone et al. (1997)
Chickpea	cry1Ac	Pod borer	Kar et al. (1997)
Sugarcane	cry1Ab	Shoot borer	Arencibia et al. (1997)
Rice	cry1Ab	Yellow stem borer, striped stem borer	Datta et al. (1998), Shu et al. (2000), and Ye et al. (2001)
	cry1Ac	Yellow stem borer	Nayak et al. (1997)
	cry2A-1Ac-gna	Rice weevil, rice hispa	Maqbool et al. (2001) and Loc et al. (2002)
	cry1B – 1Aa	Yellow stem borer	Raina et al. (2002)
	cry1Ac – 2A	Yellow stem borer, rice leaf folder	Bashir et al. (2004)
	Hybrid cry1Ac and cry 1Ab	Lepidopteran insects	Wang et al. (2010)
Oilseed rape	cry1Ac	Lepidopteran insects	Halfhill et al. (2001)
Cabbage	cry1Ab	Lepidopteran insects	Bhattacharya et al. (2002)
Cauliflower	cry1Ab	Lepidopteran insects	Chakrabarty et al. (2002)
Castor bean	cry1Ab	Lepidopteran insects	Malathi et al. (2006)
Pine	cry1Ac	Lepidopteran insects	Barraclough et al. (2009)
Sugerbeet	cry2A, cry1C	Lepidopteran insects	Litvin et al. (2014)
Jatropha	Hybrid cry1Ac and cry 1Ab	Lepidopteran insects: tortrix moth (*Archips micaceanus*)	Gu et al. (2014)
Norway spruce	cry3A	Spruce bark beetle	Briza et al. (2013)
Soybean	cry1A	Lepidopteran pests	MacRae et al. (2005)

90 % were small resource poor farmers in developing countries) grew GM crops. These adoptions of genetically modified (GM) crops improved small and marginal farmers economic status in India by USD 14.6 billion, additionally farmers benefited enormously from at least a 50 % reduction in the number of insecticide applications, thereby reducing farmer exposure to insecticides, and importantly contributed to a more sustainable environment and better quality of life (James 2013). The GM Bt crops, which are approved as commercial trait are cotton (40 events), eggplant (1 event), maize (110 events), poplar (2 events), potato (30 events), rice (3 events), soybean (4 events) and tomato (1 event) (Table 18.5).

In India, intensive efforts are under way to introduce cry genes into crop plants such as rice, potato, cotton, sorghum, check pea and vegetables. Transgenic crop species such as rice, check pea, eggplant, tomato etc., carrying different cry genes, are at various stages of research and development. India, the largest cotton-growing country in the world, reported 54,000 farmers growing 50,000 ha of Bt cotton in 2002. In 2007, the Bt cotton area had soared to 6.2 million ha grown by 3.8 million small and resource-poor farmers, which was further increased to 15 million ha in 2013 because of the significant benefits it offers (Fig. 18.4). Bt cotton has increased yield by up to 50 %, reduced insecticide sprays by half, with environmental and health implications, and increased income by up to USD 250 or more per hectare, which has contributed to social benefits and the alleviation of their poverty. Coincidentally, new biotech products such as Bt eggplant, an important food and cash crop that can benefit up to two million small and resource-poor farmers, is in advanced large scale field trials, with expectation of approval in the near term.

Adoption of Bt eggplant hybrids in India would provide a yield gain of 37 % and a reduction in total insecticide use of about 42 % over non-Bt hybrids, and increase the net returns to INR 44,117/ha (Kumar et al. 2011). The development of Bt eggplant began in 2000, and took some 9 years to complete all studies, including hybrid development, field trials, pollen flow studies, acute oral toxicity, greenhouse evaluation etc. The Genetic Engineering Approval Committee (GEAC) in India cleared Bt eggplant for commercialization on 14 October 2009. However, due to the environmental and health concerns raised by some scientists, farmers and anti-GM activists, the Government of India banned Bt eggplant on 9 February 2010. In contrast, Bangladesh has approved a Bt eggplant for planting for the first time in 2013 and serves as an exemplary model for other small poor countries. Hopefully, after more studies on GM trails, biosafety and public concerns, Bt eggplant will be released in India in the near future by responsible authorities and accepted as food without destroying biodiversity and without posing any health hazard.

18.14 Insect Resistance to Bt

Engineering with Bt genes for insect resistance in crops has been a commercially-successful technology. The crystal toxins belonging to the Cry1A group have been the most widely-used proteins in the generation of transgenic crops to combat the

Table 18.5 Country-wise transgenic crop hectarage in 2013

Country	Area (million ha)	GM crops
USA	70.1	Maize, soybean, cotton, canola, sugar beet, alfalfa, papaya, squash
Brazil	40.3	Soybean, maize, cotton
Argentina	24.4	Soybean, maize, cotton
India	11.0	Cotton
Canada	10.8	Canola, maize, soybean, sugar beet
China	4.2	Cotton, papaya, poplar, tomato, sweet pepper
Paraguay	3.6	Soybean, maize, cotton
South Africa	2.9	Maize, soybean, cotton
Pakistan	2.8	Cotton
Uruguay	1.5	Soybean, maize
Bolivia	1.0	Soybean
Philippines	0.8	Maize
Australia	0.6	Cotton, canola
Burkina Faso	0.5	Cotton
Myanmar	0.3	Cotton
Spain	0.1	Maize
Mexico	0.1	Cotton, soybean
Colombia	0.1	Cotton, maize
Sudan	0.1	Cotton
Chile	<0.1	Maize, soybean, canola
Honduras	<0.1	Maize
Portugal	<0.1	Maize
Cuba	<0.1	Maize
Czech Republic	<0.1	Maize
Costa Rica	<0.1	Cotton, soybean
Romania	<0.1	Maize
Slovakia	<0.1	Maize

Source: James (2013)

problem of lepidopteran insect pests (Jouanin et al. 1998). Continued use of a single Bt protein in crop plant will lead to resistance development in insect pests (Table 18.6). Large-scale deployment of a single Bt gene in crops imposes a continuous selection pressure in insect pests. McGaughey (1985) reported resistance development in Indian meal moth populations from grain storage bins treated with Bt formulation. Resistance to Bt insecticides was reported from field populations of *Plutella xylostella* (Tabashnik et al. 1990). Tabashnik (1994) reported development of several mutants of a variety of insects resistant to different δ endotoxins. Gould et al. (1997) gave the first direct estimate of field frequency of Bt resistant insects and reported that in *Heliothis virescens* 1 in 350 individuals carried an allele for resistance to the Bt toxin. Gahan et al. (2001) have identified a recessive gene that

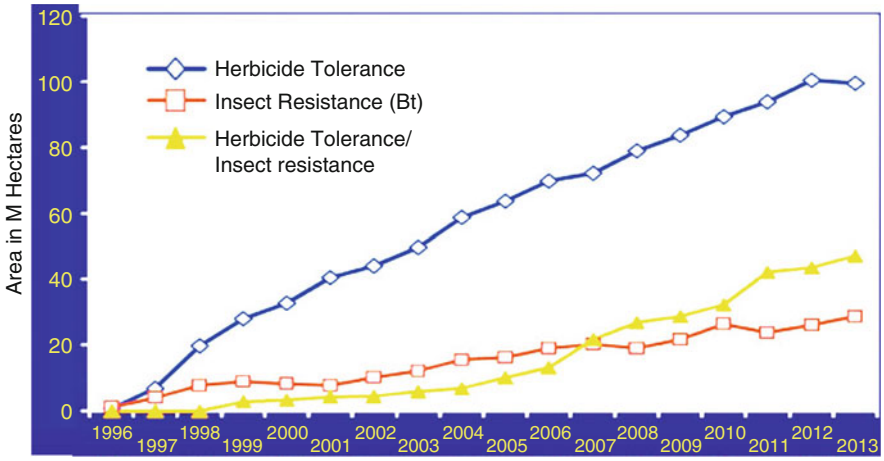

Fig. 18.4 Area of GM crops (Source: James 2013)

Table 18.6 Classical examples of insect pest and nematodes resistance to Cry toxins

Scientific name	Resistance to cry toxins	Mechanism of resistance	Reference
Caenorhabditis elegans	Cry5B	Defects in glycolipid synthesis	Griffitts et al. (2005)
Culex quinquefasciatus	Cry4A, Cry11Aa	Unknown	Georghiou and Wirth (1997)
Diatraea saccharalis	Cry1Ab	Unknown recessive	Huang et al. (2007)
Ephestia kuehniella	Cry1A, Cry2A	Tolerance owing to overproduction of lipophorin	Rahman et al. (2004, 2007)
Helicoverpa armigera	Cry1Ac	Lack of cadherin receptor, over production of esterases	Yang et al. (2007) and Ma et al. (2005)
H. zea	Cry1Ac	Unknown	Anilkumar et al. (2008)
Heliothis virescens	Cry1Ac, Cry2Aa	Lack of cadherin and alkaline phosphatase receptors. Defects in proteases	Jurat-Fuentes and Adang (2004)
Pectinophora gossypiella	Cry1Ac, Cry1Ab	Lack of cadherin receptor	Morin et al. (2003)
Plodia interpunctella	Bt ssp. Entomocidus	Defects in midgut proteases	Oppert et al. (1997)
Plutella xylostella	Cry1Ac, Cry1Ab	Unknown, recessive	Baxter et al. (2008)
Spodoptera exigua	Cry1C	Lack of aminopeptidase	Herrero et al. (2005)
Trichoplusia ni	Cry1Ac	Unknown, recessive	Janmaat and Myers (2003)

Source: Bravo and Soberon (2008)

confers much of the resistance to Bt toxin in the tobacco budworm *H. virescens*. Resistance to Cry1Ac δ endotoxin of Bt in a laboratory selected strain of *Helicoverpa armigera* was also reported (Kranthi et al. 2000). With the increasing use of Bt crops, particularly in less regulated crop systems where refuges are not required, insect resistance is likely to become an increasing problem. Therefore, ways to counter insect resistance need to be developed and put in place soon.

18.14.1 Mechanisms of Insect Resistance

Insect resistance threatens long term and continued efficacy of Bt toxins. Several mechanisms of resistance to Bt toxins have been proposed (Bravo and Sobeeron 2008; Ferre and Van Rie 2002; Gill et al. 1992; Pardo-López et al. 2013). In theory, resistance to activated Cry toxins could occur by insect pests inhibiting or blocking any step in the mechanism of action described earlier in this chapter. The different mechanisms which are mainly responsible for resistance are altered activation of Cry toxins by midgut proteases, sequestering the toxin by glycolipid moieties or esterases, by inducing an elevated immune response and by alteration of receptors resulting in reduced binding to insect gut membranes.

Two Bt resistant strains of *Plodia interpunctella* were found to lack a major gut proteinase that activates Bt protoxins (Oppert et al. 1997). Forcada et al. (1996) reported slow degradation of protoxin and quick processing of active toxin in the resistant strains of *Heliothis virescens* than in the susceptible strain. Toxin binding to BBMVs has been reported to be linked to mutation in toxin receptors such as aminopeptidase N, alkaline phosphates and cadherin receptors or mutations in other molecules such the ABCC2 transporter in lepidopteran larvae or mutations affecting glycolipid biosynthesis in *Caenorhabditis elegans*. The ABCC2 protein in its open state, where some hydrophobic surfaces of the channel are exposed to the outside, it has been hypothesized that this binding interaction could facilitate membrane insertion of the toxin oligomer (Gahan et al. 2010). In the USA, *H. virescens* is a major target of transgenic cotton expressing the Bt cry1Ac gene. Alteration of receptor, a 170 kDa aminopeptidase N is implicated as a mechanism for *H. virescens* resistance to Cry1A toxins (Jurat-Fuentes and Adang 2004). Tabashnik et al. (2000) observed that two strains of pink boll worm, *Pectinophora gossypiella* selected in the laboratory for resistance to Bt toxin Cry1Ac had substantial cross-resistance to Cry1Aa and Cry1Ab, but not to Cry1Da, Cry1Ea, Cry1Ja, Cry2Aa or Cry9Ca. The narrow spectrum of resistance and cross-resistance to activated toxin crystals suggests that reduced binding of toxin to midgut target sites could be an important mechanism of resistance. It has also been reported that some resistant insects release lipid particles containing gylcolipids into the gut lumen, which bind to Cry1Aa and Cry2Ab toxins resulting in toxin sequestration in the gut lumen, and hence affect the interaction of Cry toxins with specific receptors (Ma et al. 2012). Gunning et al. 2005 reported that sequestration of Cry1Ac in resistant *Helicoverpa armigera* is also due to the binding of the Cry toxin to esterases.

18.14.2 Resistance Management Strategies

Several strategies have been proposed to manage resistance development in insects to Bt toxins are summarized below.

Rotations Rotations or alteration of Bt toxins, insecticides and cultural or biological control strategies is the simplest approach in resistance management. Susceptibility of target pests to Bt toxin is restored when selection pressure is discontinued or changed to another gene, toxin or insecticides. However, rotations among toxins that confer cross-resistance to each other have limited value (Kumar et al. 1996).

Gene Pyramiding Multiple toxin genes with a different mode of action can be used to avoid occurrence of cross-resistance. Bosch et al. (1994) generated hybrid genes composed of cry1C and cry1E by in vivo recombination of Bt. Mandaokar et al. (1998) reported on a fusion gene coding for two different δ endotoxins belonging to classes cry1Ab and cry1B of Bt toxic to *Plutella xylostella* and useful for resistance managements. The combination of cry2Ab and cry1Ac genes in Bollgard II™ gave superior control of lepidopteran pests (Perlak et al. 2001). Maqbool and Christou (1999) transformed rice with genes for the Cry1Ac and Cry2Aa toxins and found several lines in which the levels of both toxins were more than 0.5 % of total soluble leaf protein. In SmartStax™, Bt Corn developed by Monsanto and Dow AgroSciences containing multiple genes such as Cry1F along with Cry2Ab and Cry1Ab.105 (a hybrid toxin containing domain I and domain II of Cry1Ab protein; domain III from Cry1F and the C-terminal region from Cry1Ac protein) as well as two Cry toxins active against coleopteran insects (Cry34Ab/Cry35Ab and Cry3Bb) for resistance against multiple insect pests.

High Dose Strategy A high dose of Bt toxins kills the heterozygotes allowing homozygote insects to survive. This approach maintains that constitutive and continuous expression of Bt toxins in transgenic plants may be sufficient to kill all of the heterozygotes in a population. An extremely high dose or ultra high dose is possible when Bt expression in transgenic plant is very high (1 % of total protein).

Use of Tissue Specific or Inducible Promoters This is done to avoid continuous selection pressure by achieving spatial or temporal variation in the expression levels of the Bt toxin in transgenic plants (Gujar et al. 2000).

Use of Temporal or Spatial Refuges Refuges are non-Bt crop plants that serve to maintain Bt-susceptible insects in the population (Cohen et al. 2000). Rotation of crops Bt with non-transgenic crops is a temporal refuge (de Maagd et al. 1999). In spatial refuge, part of a field is set aside for non-transgenic plants. Tang et al. (2001), based on the greenhouse tests using Bt transgenic broccoli plants expressing cry1Ac, reported that resistance of diamond back moth, *Plutella xylostella*, could be delayed by increasing the proportion of refuge plants and separate refuges delayed resistance better than mixed refuges.

A very attractive resistance management tactic is the combination of a high-dose strategy with the use of refugia (toxin-free areas). The principle is to express Cry toxins at such a dose that nearly all heterozygotic carriers of resistance alleles will be killed. Survivors would most likely mate with the sensitive insects harbored in the nearby refuge. Second generation heterozygous insects will be again killed by the toxin in the plant. Thus high dose strategy coupled with refugia would lead to more durable resistance. Consequently, a population of homozygous resistant insects would be unlikely to emerge (Ranjekar et al. 2003). In working towards the development of insect pest-resistant transgenic crops, the following may be important:

(a) Critical evaluation of the target pest, its biology and susceptibility to a range of insecticidal proteins (Bt and non-Bt sources).
(b) Selection of two or more effective toxins based on their efficacy, mechanism of action and receptor binding.
(c) Evaluation of the biosafety of insecticidal proteins.
(d) Optimization of gene expression as evidenced by studies in model systems like tobacco.
(e) Selection of suitable and effective promoters based on spatial and temporal aspects of insect infestation.
(f) Expression of multiple insecticidal genes driven by different promoters in transgenic crop of interest (either via co-transformation or by plant breeding).
(g) Selection of the transformed plants with single copy transgene insertion and high levels of toxin expression (Kumar 2004).

18.15 Effect of Various Farm Management Practices on Bt Toxin Production in Transgenic Crops

Huang et al. (2014) reported the effect of various agronomic practices on Bt protein production in Bt cotton fields in China, which indicate that toxin production is highly affected by cotton genotypes as well environmental conditions. Huang et al. (2014) reported that the Bt toxin expression levels decrease by more than 50 % from early growing season to late growing season, hence correct sowing is important to avoid any crop damage because of pest infestation. The Bt cotton seeds saved by farmers showed less toxin expression compared to the brought seeds, hence the recommended practice should be bought seeds. Further analysis from Huang et al. (2014) suggested that applications of phosphate and potash fertilizers and manure will increase the Bt toxin expression significantly, however application of nitrogen fertilizer has no significant effect on expression of Bt toxin in the farm field. However, in most parts of the world, farmers use more nitrogen, less phosphorus and potash, which will not be beneficial in the case of transgenic Bt crops. Hence, balanced fertilizer use not only contributes to crop production, but also the expression of Bt toxin which leads to higher crop yield and lower pesticide cost.

18.16 Bt Beyond a Potent Insecticide

The insecticidal proteins produced by Bt have provided a uniquely specific and effective tool for the control of a wide variety of insect pests. However the other roles of Bt proteins have not been paid great attention. The Bt crystal protein, parasoprin, is capable of killing cancer cells, makes this protein as possible candidate for anticancer agents of medical use (Saitoh et al. 2006). Zwittermicin A, a linear aminopolyol antibiotic produced by Bt, has very high activity against the oomycetes and other fungus as well as some Gram-negative bacteria (Zhao et al. 2007). The Bt crystal protein Cyt1Aa has an antibacterial effect on pathogenic bacteria (Cahan et al. 2008). Different bacteriocins produced by Bt have a broad spectrum of biological activities against important pathogenic bacteria (Fuente-Salcido et al. 2008). Antimicrobial peptide, Thuricin-17 (bacteriocin) from Bt stimulates plant growth and can be potentially used as a plant growth promoting rhizobacteria (PGPR) (Lee et al. 2009). Crystal proteins from Bt are also effective against human pathogenic protozoan *Leishmania* which suggests that Bt is a safe therapeutic antileishmanial agents (El-Sadawy et al. 2008). Acyl homoserine lactone lactonase produced by Bt can hydrolize N-acyl homoserine lactone, a signal molecule of some plant pathogenic bacterial quorum-sensing systems and significantly silences bacterial virulence by quenching bacterial quorum sensing (Park et al. 2008). Jain et al. (2009) reported a simple protocol for preparation of silver nanoparticles using Bt spore crystal mixture.

18.17 Transgenic Plant Breeding in Relation to Bt

The main objective of plant breeding is development of genetically-improved plant varieties with desirable traits. Apart from traditional breeding processes, plant breeding also employs gene transfer or genetic engineering as an additional tool for improving crops. Transgenic breeding is an extension of plant breeding technologies, which offers two unique breeding opportunities; one is to introduce novel genetic variation that is not available in the current breeding germplasm pool and the other is to create desired phenotypes from known genes (Zhong 2001). For a successful transgenic breeding program, the primary requirement is to generate a large number of independent transgenic plants (each independent transgenic event arises from a single engineered cell with a single copy of the gene, commonly referred to as an event), each event performs differently in the field because of positioning effects and out of which he best can be selected. Furthermore, such transgenic plant (single event) or transgene/transgenes (gene staking) of interest coding for an agronomic trait are then integrated into elite germplasm through breeding (Lowe et al. 2009). Zhong (2001) summarized three desired characteristics of transgenic plants after initial selection which need to be taken when evaluating transgenic phenotypes. First, the transgenic trait needs to have high efficacy in field conditions, Second, the transgene(s) need to follow Mendelian segregation at every generation, which should be verified at the T2 generation and have high stability

under various environmental conditions and third, the transgene should not insert into a known gene which could have a deleterious effect or negative effect on the plant phenotype, and therefore its agronomic worth.

While carrying out genetic transformation in crop plants, most laboratories use non-elite genotypes as transformation procedures, are genotype specific and also many elite genotype do not respond well (Visarada et al. 2009). Mumm (2007) summarized transgenic breeding in: (1) Forward breeding can be employed to derive new lines to create better hybrids or breed new lines while simultaneously introgressing transgenes, as with outcrossing species. (2) If elite hybrids are already developed, backcross breeding can integrate transgenes into the recurrent parent while minimizing the chromatin of the donor parent. (3) Combinations of both backcross and forward breeding programs can be run in parallel for gene pyramiding/gene staking.

Knowledge of practical breeding can help guide the choice of appropriate strategies for developing transgenic products. Monsanto markets cry1Ac cotton (event 531), which is transferred to local germplasm in several countries. In India, MAHYCO in collaboration with Monsanto introduced the modified cry1Ac gene originally used to transform the Coker 312 variety of cotton into parental lines of hybrids of Indian cotton through back-crosses. These hybrids were field evaluated at different locations. Various experiments related to environmental and toxicological studies e.g. gene flow, effects of pollen and plants on non-target organisms, etc. were conducted and based upon the positive results, in 2002, the Government of India approved commercial release of Bt-cotton and India received its first transgenic crop. Until now, none of the commercial transgenics Bt cotton in the country are transgenic regenerative plants, rather they were bred between transgenic regenerative plants and commercial varieties.

Liu et al. (2010) reported the introgression of three Bt genes such as cry1Ac, cry1C and cry2A to the rice elite gernplasm 9311 and Fuhui 838 from the donor parents, respectively, by the molecular maker selection approach. Gao et al. (2013) reported that transgenic plants developed through genetic transformation and by backcrossing or marker assisted selection are equivalent at both molecular and biological levels in terms of performance.

18.18 Conclusions and Prospects

The global human population reached ~7.5 billion in 2014 and is expected to reach approximately to 8.5 billion by 2025. Thus, to meet the increasing needs of the increasing population, it will be necessary to produce 50 % more food by 2025 for which crop varieties with higher yield and stability are required. Thus, the major challenge is how to increase and sustain crop productivity with less use of pesticides and this lies in Bt, which is 300 times and 80,000 times more toxic to synthetic pyrethroids and organophosphates, respectively. The commercial use of Bt as a suspension of spores and inclusions has been limited in part due to the need to spray at rather frequent intervals, in order to sustain an effective level of biopesticide. This problem has been circumvented by engineering plants (cotton, maize etc.)

to producing Bt toxin in the plant system. Transgenic Bt-cotton expressing Cry1Ac has been registered for commercial cultivation in India in 2002 and it primarily targets the cotton bollworm *Heliocoverpa armigera*. However, continued use of a single Bt protein in GM crops such as Bt-cotton will lead to resistance development in lepidopteran insects. An effective way to overcome the problem of resistance development is cloning of novel cry genes with increased insecticidal activity due to sequence variations. Furthermore, such toxins can be improved by protein engineering (DNA shuffling) so that they can be used effectively in plant transgenesis. Also, more knowledge and understanding of the receptor in the insect pest and the mode of action of different novel and engineered toxins will help in developing new, more efficient Bt crops and spray products. Therefore, we anticipate a brilliant future in the use of Bt Cry proteins to control important insect pests in agriculture and reduce the use of chemical pesticides, which will also have a positive impact and lead to a healthier environment.

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