Aditya Pratap · Jitendra Kumar *Editors*

Alien Gene Transfer in Crop Plants, Volume 2

Achievements and Impacts

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Foreword

The book "Alien Gene Transfer in Crop Plants: Achievements and Impacts" is a meticulously compiled volume by Aditya Pratap and Jitendra Kumar, the two young agricultural scientists, who deserve appreciation for their efforts. This volume offers an extensive reference on the developments made through conventional as well as modern alien gene transfer practices in major agricultural crops of the world including cereals, pulses, oil crops, sugarcane, and vegetables. The chapters in this book have been contributed by well-known scientists who are recognized globally for their scientific contributions. I hope that the information contained in this book will be useful to researchers, teachers, and students in providing an insight into the subject and will further encourage them to go deeper into this field to solve the problems of food and nutritional security.

2013 marks the 60th anniversary of the elucidation of the double-helix structure of the DNA molecule, the chemical substance of heredity, 60 years ago by James Watson, Francis Crick, Maurice Wilkins, and Rosalind Franklin. This finding has opened up uncommon opportunities for the advancement of science as related to all aspects of life. During recent decades, many Nobel Prizes in Physiology and Medicine have gone to Molecular Geneticists. At the same time, public concern about the proper measurement of risks and benefits has grown, particularly in the

fields of agricultural and food biotechnology. Biotechnology provides an opportunity to convert bioresources into economic wealth. This has to be done in a manner that there is no adverse impact either on the environment or on human and animal health. The bottom line of our national agricultural biotechnology policy should be the economic well-being of farm families, food security of the nation, health security of the consumer, protection of the environment, and security of our national and international trade in farm commodities. In view of the importance of alien gene transfer in facing the problems of the future such as expanding biotic and abiotic stresses and climate change, I will like to refer to some of the broader implications of recombinant DNA technology.

Medical biotechnology—extending the length and quality of life: Medical biotechnology is helping to extend human longevity as well as quality of life. Gene therapy offers promise in curing diseases like leukemia and promises to open the door to the cure of diseases like Parkinson's and Alzheimer's. Research in the area of medical biotechnology needs to be strengthened, particularly with reference to diseases like malaria and tuberculosis. Human and animal health are closely interrelated. India has emerged as a global hotspot of zoonotic diseases due to the close proximity of farm animals and people. More interdisciplinary studies are needed on the economic and nutritional implications of zoonosis.

Environmental biotechnology—bioremediation: Pollution problems are becoming serious in the case of both river and groundwater. In India, the arsenic content of groundwater is increasing at several places, particularly in West Bengal. Tamil Nadu's two major industries, namely, leather and textile, cause water pollution. Hence, there is need for a considerable step up in bioremediation research. A coordinated project, organized on a mission mode, is necessary to harness the tools of biotechnology for water purification and for providing safe drinking water.

Agricultural and food biotechnology: Unlike in the case of medical and environmental biotechnology, there are concerns about the risks and benefits in the area of crop biotechnology. While molecular marker-assisted breeding is considered safe, the potential adverse impact of genetic modification in crops, involving recombinant DNA technology, is an area of wide divergence of opinion not only among the public but also among professionals. The precautionary principle is widely advocated in assessing the risks and benefits and to ensure risk avoidance. A well-designed *need assessment* procedure should be introduced to facilitate decisions relating to the choice of problems for genetic modification research as well as for prioritization in the investment of public funds. While assessing needs and fixing priorities, the rich diversity of agro-ecosystems and socioeconomic conditions should be kept in view. There is also need for a considerable strengthening of public good research so as to ensure social inclusion in access to desirable technologies.

The Department of Biotechnology, Government of India, on the basis of widespread consultations has developed a draft bill for the establishment of a Biotechnology Regulatory Authority. This bill was introduced in the *Lok Sabha* on 22nd April 2013 by the Union Minister for Science and Technology. Earlier, the Parliamentary Committee on Agriculture chaired by Shri Basudeb Acharya had recommended "to

assess risks and benefits from GMOs with reference to biodiversity, human and animal health and environment, a National Biosafety Authority is needed."

Since the Draft Act has already been introduced in the Parliament (of India), I presume that it will be examined by an appropriate committee of both Houses of Parliament. During this process, the various suggestions made by the Basudeb Acharya Committee as well as Civil Society Organizations could be examined carefully and a suitable title for the bill could be chosen. Also, it is clear that the Parliament-approved Bill should help to ensure that the risks and benefits associated with GMOs are assessed in a scientifically credible and transparent manner. The regulations must be based on the best available science and also wherever necessary on the precautionary principle, using the Cartagena Protocol on Biosafety as the guiding internationally approved protocol.

Biosafety and bioethics—capacity building and information empowerment: Capacity building efforts should start with the *Gram Panchayats* and extend to school and college students and all those who will be involved in biotechnological enterprises. There is need for greater genetic literacy among farmers as well as the general public. The ongoing programs for public and political information should be strengthened. For this purpose, a Committee on Public Understanding of Science could be set up under the Government of India Media Resource Centres in local languages for the purpose of providing authentic scientific information relating to molecular genetics and biosafety. Advanced training programs in biosafety and bioethics should be organized in appropriate universities/institutions.

Harnessing the power of partnership: The new genetics offers immense opportunities for collaboration among scientists belonging to different disciplines and working in different institutions. International collaborations as well as public–private partnership should be fostered wherever this will help to achieve the desired applied goals surely, speedily, and economically. All such partnerships should be based on a well-defined ethical code which takes into account issues like conflict of interest and IPR. Public good research should be promoted actively by public-funded institutions and universities, so that the new technologies reach the unreached. Farmer participatory research should be promoted, based on respect for community rights.

Every farm a nutri-farm—role of biofortification: Considering the widespread prevalence of both undernutrition and hidden hunger caused by the deficiency of micronutrients like iron, iodine, zinc, vitamin A, and vitamin B12 in the diet, it would be useful to promote the integration of nutritional criteria in the farming systems currently adopted by farm families. Biofortification, both naturally occurring and achieved through breeding, offers scope for providing agricultural remedies to the nutritional maladies prevailing in the area. The initiative of the Government of India in promoting nutri-farms and for including a wide range of local grains (nutri-cereals) under the Public Distribution System (PDS) in the National Food Security Bill are welcome steps.

I congratulate the authors as well as Dr. N. Nadarajan, Director of Indian Institute of Pulses Research, for this very valuable contribution. Now that food has become

a basic human right (in India) through the National Food Security Act 2013, there is no time to relax on the food production front. We must utilize both classical and contemporary technologies in improving the productivity, profitability, and sustainability of small holders. I therefore hope that this book will be widely read by scholars and scientists as well as policy makers.

Chennai, TN, India M.S. Swaminathan

Preface

Alien gene transfer in crop plants has emerged as a boon to humanity as well as science. Since the beginning of plant breeding as a systematic endeavour, scientists have spent a major proportion of their energy, time and resources in planning and executing sexual hybridizations with an objective to create additional genetic variability hitherto not available in nature, followed by selections of desirable recombinants to develop improved genotypes which would be even more beneficial to mankind. After the rediscovery of Mendelism and subsequently our improved understanding of plant traits, their genetic control and inheritance, interest started growing towards transferring genes conferring traits of interest from distant and wild relatives, and even from across genome boundaries.

Wild crop genetic resources are rich reservoirs of useful alien genes. These have contributed tremendously in unleashing the basic and fundamental questions of life including those on origin, history and evolution of crop plants, their phylogenetic relationships and inheritance of simple as well as complex traits. Some of them have even served as excellent model plant species, helping to resolve several mysteries associated with crop flora as well as elucidation and interpretation of several plant species genomes. They also provided numerous donors for genetic improvement of the cultivated types providing sources for disease and insect-pest resistance, resistance/tolerance to climate extremities and problem soils, improved quality traits and keeping quality, and biofortification. During the progression of alien gene transfer technologies, several novel concepts and theories, for example, doubled haploid breeding, were also promoted. Encouraged by the success of alien transfers in genetic amelioration of crop plants, the researchers started looking for alien genes even across genome boundaries and devised horizontal gene transfer strategies, genetic transformation becoming one of the most powerful tools in changing our lives and way of living. The impact of genetic transformation can be realized from the fact that the development, application, and socio-economic and political implications of transgenic crops now affect the agrarian policies and economies of several countries despite the fact that a large group of environmentalists, scientists and consumers is highly sceptic about their use and after effects. Nevertheless, even

severe criticism could also not deter the researchers in furthering their quest for newer genes and prompted them to come out with the concepts of cisgenesis and intragenesis to address the concerns of those worried about the safety and utility of GM crops. All these alien gene transfer techniques, aided by in vitro procedures, hormonal manipulations, polyploidization and mutation, and of late, molecular marker technology and precise detection of alien genes through molecular cytogenetics have led to introgression of hundreds of genes of interest in cultivated backgrounds of crop plants, thereby improving their genetic potential for yield, quality and economic viability. The introgression of *Lr* genes in wheat, development of high-yielding and input-responsive rice and maize, noblization of sugarcane and development of *Bt*, Flavr Savr and Roundup Ready GM crops are just a few of the glorious examples of how alien gene transfer can revolutionize the global agriculture. Some of the early developments in alien gene transfer, of course most of them achieved through conventional breeding, coupled with better agronomic inputs and irrigation triggered the historical "Green Revolution," which changed the lives and fate of millions of people in the tropical wetlands of developing and newly industrializing countries such as India, Pakistan, Indonesia, Bangladesh and China.

Fascinated by the miracle of alien gene transfer in crop plants and revisiting the green revolution of India several times during the course of our study and research, we initiated prebreeding work in two important food legumes of the Indian subcontinent, *Vigna* and lentil, when we joined our current position at the Indian Institute of Pulses Research, Kanpur. However, it was only then when we realized that alien gene transfer, whether vertical or horizontal, is associated with numerous promises and opportunities and even more problems and constraints. As we proceeded with the gene transfer procedures in our respective crops, each step required a lot of stopgap, discussions and relook into the literature, more so because hybridization, in general, is difficult in pulses and also, these are recalcitrant to in vitro techniques. We had to often look into so many periodicals, journals, reports, and research notes and search internet for long before we could find suitable literature helping us or even sometimes, nothing was found at all. We realized that while some crops have been tremendously benefited from alien gene transfer, in other crops it could not have been successful at all. It is then when we conceived the idea to bring the most relevant information on alien gene transfer at one platform so that the academicians, researchers and post-graduate students in agriculture and biology have a ready reference with them on the most important aspects of alien gene transfer. Nonetheless, the scope of this subject is so vast that we decided to go with two volumes; one on the theoretical aspect of innovations, methods and risks associated with alien gene transfer and the second one, of more practical nature, on the achievements and impacts of alien gene transfer, covering mainly the agricultural crop plants, which is the area of our specialization. The first volume is already available, and probably you might have got a chance to read it.

The second volume is now in your hands and has been divided into four sections dealing with cereals, pulses, oil crops and some other important crops. Cereals is the group that has been benefitted most from alien gene transfer. The first section describes achievements and impacts of alien transfer in cereals covered in first five

chapters, one each on wheat, barley, corn, oats and pearl millet. Achievements through alien gene transfer have been variable in pulses owing to difficulties as mentioned earlier and the next section describes pulses in subsequent four chapters (Chaps. 6–9) each on four most important pulse crops viz., chickpea, pigeonpea, *Vigna* species and lentil. The third section is on oil crops and elaborates *Brassica*, oilpalm and coconut, groundnut and sunflower spread over four chapters from Chaps. 10–13. The last section covers sugarcane, one of the most important commercial crops (Chap. 14) and two important vegetable crops of the Solanaceae family viz., tomato and eggplant (Chaps. 15 and 16). All the chapters have been well supported by classical as well as current references, tables and colourful illustrations, wherever necessary.

For this volume, initially we had planned to include a few more crops in each section. However, we had to exclude some of the topics later due to either very less work done on this theme in some of the crops or a few authors not responding at the last moment leaving no time with us to make alternative arrangements. A few authors also had delayed manuscript submissions due to some unavoidable personal or professional circumstances. Nevertheless, all scientists who finally contributed to this volume are well recognized and accomplished researchers in their fields and we sincerely thank all of them for writing their chapters meticulously and with great zeal and responsibility.

We will be failing in our duties if we do not convey our heartfelt gratitude to the people who have directly or indirectly contributed in successful completion of this volume. First of all, the authorities in the Indian Council of Agricultural Research (ICAR), New Delhi, Dr. S. Ayyappan, Secretary, Department Agricultural Research and education (DARE), Government of India and Director General, ICAR; Prof. Swapan Kumar Datta, Deputy Director General (Crop Science), ICAR and Dr. B.B. Singh, Additional Director General (Oilseeds and Pulses) deserve our heartfelt thanks for providing us state-of-the-art facilities for furthering our research and academic pursuits, especially in the field of prebreeding and alien gene transfer. We are extremely grateful to Prof. M.S. Swaminathan, the living legend and popularly known as the "Father of Green Revolution" in India for blessing us for the success of our scientific endeavors and writing the preface of this Volume. The name of Dr. N. Nadarajan, Director, Indian Institute of Pulses Research deserves a special mention for being a driving force in motivating us to undertake this endeavour. We are also grateful to our colleague Debjyoti Sen Gupta and the research scholars working with us, Nupur Malviya, Rakhi Tomar, Ekta Srivastava and Mrityunjaya Singh, for their help in compilation of references, typing some of the materials and searching voluminous literature related to the topic. The entire team at Springer, especially Hannah Smith, Mellisa Higgs and Kenneth Teng, the commissioning editors and Daniel Dominguez, the developmental editor, have always been cooperative and helpful during the preparation of this volume and deserve our genuine appreciations. Their thorough professional approach appreciating our difficulties and being accommodative for the last-minute changes deserve special acknowledgements. Our lovely kids Puranjay, Neha and Gun always kept us going with their charming smile and their childish freshness and a

silent approval to tax on their time is deeply appreciated. Above all we feel it our proud privilege to acknowledge Dr. Rakhi Gupta and Mrs. Renu Rani, our life partners, who despite their own professional and domestic commitments, allowed us to work overtime and meanwhile took care of all household and personal responsibilities which we were supposed to shoulder.

It is our sincere hope that this book will be a useful knowledge resource to the researchers, students and scholars who are involved in teaching, research and studies of gene transfer in crop plants and development of new crop varieties for the betterment of mankind. Nothing than the words of Dr. Norman E. Borlaug can be better to conclude the preface of this volume who said in the first press conference after his Nobel Peace Prize was announced that "the work of his institute, and any similar work would only win us all perhaps 20 years breathing space. The potential resources of food were limited. Unless the growth of population could be controlled, then we should destroy the species." Perhaps until then we will have to keep our quest alive for always searching new genes for improving the crop plants further to support mankind.

Kanpur, India

Aditya Pratap
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Chapter 1
Wheat

Harinder Kumar Chaudhary, Vineeta Kaila, Shoukat Ahmad Rather, Anila Badiyal, Waseem Hussain, Navdeep Singh Jamwal, and Anima Mahato

Abstract Ensuring food security in the era of climate change is of major concern to the plant breeders worldwide. Wheat, being the staple food crop of the world, needs more focus by the scientific community for its genetic upgradation and development of cultivars tolerant to prevalent biotic and abiotic stresses. The role of alien gene introgression in utilization of wild genetic resources to enhance the genetic diversity in the cultivated wheat varieties is widely acknowledged. Classical approaches of alien gene transfer like wide hybridization have been practiced to a great extent but are hindered by a number of factors like linkage drag and poor crossability among the species. When such approaches are coupled with novel biotechnological tools, they allow swift, precise and targeted gene transfer. Transgenic approach offers a great advantage in alien gene transfer by keeping aside the problems encountered in previous approaches and opens new avenues for alien gene transfer in wheat, hence ensuring broadening and diversification of wheat genome. This chapter focuses on the successful attempts of alien gene introgression into wheat through various approaches along with their limitations and future prospects.

Keywords Alien translocations • Doubled-haploid production • Direct gene transfer • Gene pool • Triticale • Wheat

1.1 Introduction

Wheat, the universal cereal of Old World agriculture, belongs to the botanical tribe Triticeae and family Poaceae (Zohary and Hopfmann 2000). The crop was domesticated around 10,000 years ago in both tetraploid and hexaploid forms

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(Dubcovsky and Dvorak 2007). Nowadays hexaploid wheat (*Triticum aestivum*), commonly known as bread wheat ($2n=6x=42=$AABBDD), provides roughly a fifth of the world's food requirement (FAO 2012). Being one of the most important and widely cultivated food crops, it provides about 20 % of the calories consumed by the humans globally (Gustafson et al. 2009). Tetraploid wheat (*T. durum*), commonly known as durum wheat ($2n=4x=28=$AABB), is used for the preparation of products like macaroni and low-rising bread. The world's major bread wheat-producing areas are in northern China, northern India, northern USA and adjoining areas in Canada, northern and central Europe, western Russia, southern Australia, southern Latin America and South Africa. Global production of bread wheat in 2011 was 704 million tonnes, with an average yield of 3.19 t/ha. The world population currently increasing by around 100 million per year is expected to exceed 10 billion by 2050, with a concomitant requirement to double the food produced from the same amount of arable land. Anticipated changes in climate and its variability, particularly extreme temperatures and changes in rainfall, are expected to make crop improvement even more crucial for food production in important crops like wheat (Lobell and Burke 2008). To ensure global, political and social stability, increasing sustainable wheat production equitably without compromising environmental integrity remains a major challenge. The best strategy for sustainable wheat improvement in the era of climatic change is to utilize the adaptive genetic resources like wild progenitors that act as reservoirs of huge genetic diversity (Nevo 2011). Within the primary gene pool, the modern plant breeding approaches have eroded the genetic diversity that increased susceptibility and vulnerability to environmental stresses, pests and diseases (Nevo 2011). Alternatively, gene introgression of favourable alleles and gene/gene complexes from wild relatives offers excellent and efficient opportunity to enhance the genetic base of cultivated gene pool for various desirable traits. The wild species of wheat are still a valuable source of useful agronomic traits for the continued improvement of cultivated wheat. Wide hybridization of wheat with grasses coupled with cytogenetic manipulation of the hybrid material has been instrumental in the genetic improvement of the crop. Chromosome engineering methodologies based on the manipulation of pairing control mechanisms and induced translocations have been employed to transfer specific disease and pest resistance genes from annual (e.g. rye) or perennial (e.g. *Thinopyrum*, *Lophopyrum* and *Agropyron*) members of the tribe Triticeae into wheat. With the advancement of biotechnological tools, transfer of targeted traits to elite wheat lines both at interspecific and intergeneric level has been made possible successfully, but it needs more care and attention due to inherent problems of integration, stability and expression of transgene (Vasil 2007).

From evolutionary point of view, wheat is a superb model organism of allopolyploid speciation, adaptation and domestication in plants (Gustafson et al. 2009). Wheat evolved through amphiploidy (Feldman and Levy 2005) and behaves like typical genomic amphiploids, that is, their chromosomes pair in a diploid fashion and mode of inheritance is disomic. Major evolutionary driving force in enhancement of genetic diversity and adaptive radiation of wheat (Feldman and Levy 2005) is its allopolyploid nature, resulting in the formation of first allotetraploid wheat species, *T. dicoccoides* (0.5 million years ago) from which the hexaploid wheat

species, *T. aestivum*, was developed around 10,000 years ago. Presence of diverse and buffering genomes in wheat increases its adaptability and rapid expansion in the world and facilitates gene introduction from other species and genera. However, alien gene introgression into cultivable varieties has to be carefully achieved and controlled. Problems like genetic recombination in homoeologous chromosomes and linkage drag are often accompanied by unacceptable wild traits causing complete or partial sterility that appear to be unavoidable constraints in alien introgression in wheat. The modest success in utilizing alien sources for wheat improvement till date may be due to the lack of proper transfer strategies. However, genetic transformation is still far from being a feasible strategy for the majority of traits of agronomic relevance. Sears (1981) described the main avenues of the chromosome engineering approach for transferring such segments of alien chromosomes carrying particular desired genes to wheat chromosomes. Chromosome engineering-mediated approaches of alien gene introgressions have gained substantial support due to advancement in hybridization techniques, chromosome engineering methodologies and biotechnological procedures. Advent of recent molecular marker techniques as well as molecular cytogenetic techniques, such as non-radioactive in situ hybridization like genomic in situ hybridization (GISH) and fluorescent in situ hybridization (FISH), can effectively complement classical diagnostic and selection tools for more efficient and accurate detection and characterization of desired products.

1.2 Gene Pool and Its Utilization

The wheat gene pool is structured upon the genomic constitution of the species and comprises three groups, viz., primary, secondary and tertiary (Fig. 1.1). Variability for some traits is limited or even exhausted within the primary wheat gene pool. Secondary and tertiary gene pools represent a wide and yet little exploited reservoir of desirable alien genes that can be incorporated into cultivated wheat genotypes. Primary gene pool includes the hexaploid landraces, cultivated tetraploids, wild *T. dicoccoides* and diploid donors of the A and D genomes of hexaploid wheat. Gene transfer from primary gene pool is straightforward and requires standard breeding methods like hybridization, backcrossing and selection and homologous recombination either through direct crosses of these species with common wheat or production of synthetic wheat (McFadden and Sears 1946; Gill and Raupp 1987). Many genes conferring resistance to diseases and pests have been transferred using these methods, and several of them are still being exploited in wheat improvement programmes (McIntosh 1991). The secondary gene pool consists of the polyploid *Triticum* and *Aegilops* species which share one genome among the three genomes of wheat. Gene transfer from secondary gene pool requires cytogenetic manipulations to enhance the recombination between alien and wheat homoeologous chromosomes.

Wild relatives with genomes that are non-homologous to wheat reside in the tertiary gene pool that includes diploid and polyploid species of Triticeae-carrying genomes other than A, B and D. As chromosome pairing and recombination in

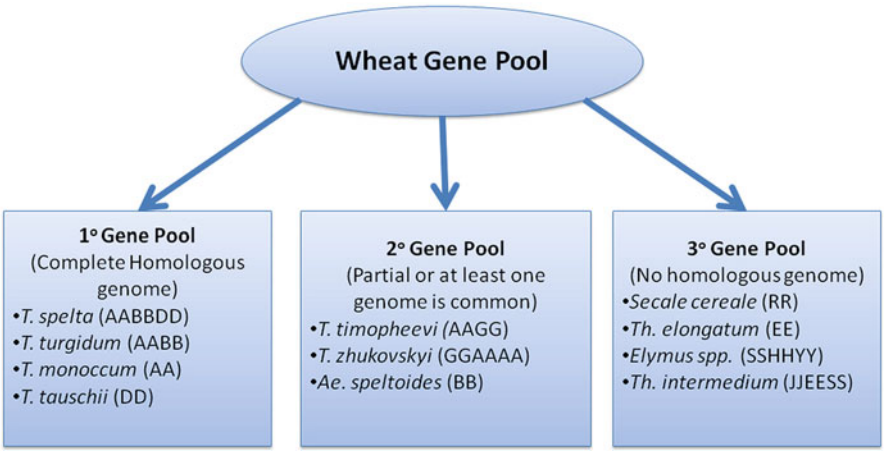

Fig. 1.1 Wheat gene pool representing various species

common wheat are largely governed by the gene *Ph1* located on the long arm of chromosome 5B which ensures pairing and recombination of homologous chromosomes only (Riley and Chapman 1958). Hence, other strategies need to be employed for utilization of these species in introgression programme as gene transfer from these species cannot be achieved through homologous recombination. Both physical and genetic methods that cause random chromosome breaks and promote recombination have been used in engineering transfers from the tertiary gene pool into the genetic background of cultivated wheat species.

1.3 Strategies for Gene Transfer

Various strategies that have been used from time to time for alien gene introgression in wheat include chromosome engineering methodologies based on the manipulation of pairing control mechanisms, genetic transformation and transgenic approaches. For alien gene introgression and effective utilization of large Triticeae gene reservoir, novel techniques must be developed that allow introgression of alien chromatin into wheat genetic background and enable selection of novel alleles without sacrificing important traits. To accomplish such goals, a breeder must understand the genomic constitution of the donor as well as the recipient species and the relationship between the two.

Pairing between chromosomes of the alien donor and cultivable wheat is the key to such gene introgressions. Hexaploid wheat with three genomes has a definite genetic control of chromosome pairing in which only homologous chromosomes pair to form bivalents and there is no pairing between homoeologous chromosomes. In hybrids between a wheat cultivar and wild relative, genetic recombination of

homoeologous chromosomes is completely suppressed due to the presence of *Ph* locus on 5B chromosome of wheat. The *Ph1* system in wheat is useful for chromosome engineering in relation to genetic enhancement of wheat.

Hence, to transfer desirable chromosomes/target gene for genetic improvement of wheat from relative species, it is compulsory to manipulate *Ph* locus so that homoeologous pairing and recombination occur freely between different genomes of wheat and alien chromosomes (Ceoloni and Donini 1993). In addition to pairing and recombination between wild segments and elite genomes for the transfer of alien chromosome segment, linkage drag that carries undesired alien genes in addition to the target gene must be avoided (Feldman and Levy 2005). For transfer and reduction of the size of alien chromatin segment transferred to a crop plant genome, different cytogenetic manipulation techniques like chromosome engineering (Sears 1981) can be adopted. The basic strategies that have been utilized for alien gene introgression in wheat from time to time can be summarized as:

- Addition of the whole alien genome through production of amphiploids
- Production of a pair of alien addition and substitution lines
- Production of a recombinant chromosome with desired gene

1.3.1 Whole-Genome Transfer

First and foremost approach for alien genome transfer is to cross the hexaploid wheat with any of its nearest relatives. All genomes of the perennial Triticeae species have been combined, either singly or in combination, with the A, B and D genomes of bread wheat (Wang 1989; Mujeeb-Kazi et al. 1994). *Aegilops* species has remained the donor of choice for researchers for its similarity with (Feldman and Levy 2005) and contribution of D genome (McFadden and Sears 1946) during the wheat genome evolution. Due to the close relationship between wheat and the *Aegilops* species, homologous recombination between the two genera occurs naturally. The appearance of spontaneous *Triticum*×*Aegilops* hybrids was observed by Leighty and Taylor (1927), and Vavilov (1935). Since then, a large number of *Triticum*×*Aegilops* and reciprocal hybrids were developed under artificial conditions (Logojan and Molnár-Láng 2000; Ozkan and Feldman 2001). The transfer of whole genome was observed in the following cases:

1.3.1.1 Triticale

The first attempt to transfer whole genome in wheat was made by Rimpau (1891) who developed the first man-made cereal, *Triticale*, after crossing wheat with rye. Genomic instability and sterility remained the major hurdle in such transfers, so the production of fertile amphiploids had to wait till the advent of colchicine treatment (Eigsti and Dustin 1955) which established triticale as a field crop. Triticale has remained the focal point for plant breeders time and again for its enviable

combination of grain quality traits of *Triticum* with the vigour and hardiness of *Secale* (Kim et al. 2003). Being the only bridging species between wheat and rye, it has attracted the plant breeders worldwide for further introgression studies.

1.3.1.2 Synthetic Hexaploid Wheat

In order to capture new diversity in already available cultivable gene pool, concept of synthetic hexaploid wheat was introduced. The development of synthetic wheat came into light in 1980s when a new synthetic cross was made using a tetraploid wheat and *Ae. tauschii* accession to increase the genetic diversity in bread wheat (Gill and Raupp 1987). China became the first country to commercialize synthetic wheat by releasing the first synthetic wheat variety "Chaunmai 42" for cultivation in 2003. Indeed, this method was suitable for gene transfer from *Ae. tauschii* to wheat and has been adopted by a larger number of wheat breeders.

In hexaploid synthetic wheat, tetraploid wheat with AB genome is hybridized with *Ae. tauschii* (DD), and the chromosomes of the F_1 hybrid are doubled using colchicine treatment. The product is a fertile synthetic hexaploid (AABBDD) genotype fully homologous to bread wheat. Hexaploid wheat homologous chromosomes will readily recombine with the hybrid. Such synthetic lines serve as a gene pool derived from *Ae. tauschii* that is ready for screening of any desired trait and allows an easy transfer of the responsible gene. Although a new arena opened with synthetic wheat-mediated introgression, certain issues like hybrid necrosis (Pukhalskiy et al. 2000) and excessive hardiness in rachis and glume pose serious problems in this direction. Moreover, from agronomic point of view, the results were not much impressive.

1.3.2 Development of Substitution, Addition and Induced Translocation Alien Lines

Transfer of traits from alien species has also been made possible through development of alien addition, substitution and translocation lines. Due to the presence of diverse genomes in wheat and triplicate nature of the genes, bread wheat easily tolerates addition or deletion of a pair of chromosomes, single chromosome or part of a chromosome, hence allowing the production of addition, substitution and deletion stocks. These lines are the initial requirements of pre-breeding programmes aimed at the transfer of alien traits to cultivated varieties (Feldman and Levy 2005). However, development of these lines often exhibits sterility. To overcome this, hybrids are made fertile either through amphidiploidy or promotion of homoeologous pairing (O'Mara 1940). Feldman and Levy (2005) and Sears (1981) independently produced alien addition and substitution lines through repeated backcrossing of the amphiploid to the wheat parent. Knowing the characters of alien species, it is possible to identify chromosomes or their segments based on plant morphology, chromosome banding and biochemical and molecular markers (Jauhar et al. 2009).

1.3.3 Engineering the Chromosome with Desired Gene

To overcome the linkage drag and reduce size of the alien chromosome segment to be transferred into wheat, certain cytogenetic manipulative approaches were developed which were collectively termed as chromosome engineering by Sears (1981). Chromosome engineering has opened new frontiers for transferring related or unrelated chromatin into the genetic background of wheat. Traditional breeding and classical cytogenetic techniques for undertaking such manipulations include modification in *Ph* locus, irradiation, use of gametocidal genes, induced homoeologous pairing, genomic reconstruction of wheat through translocations and their further characterization after in vitro regeneration. On the other hand, the transgenic approach includes direct gene transfer through protoplast uptake, particle bombardment and *Agrobacterium tumefaciens*-mediated techniques.

1.3.3.1 Induction of Homoeologous Pairing

For effective gene transfer, induction of pairing between different genomes of recipient and donor is desirable. Various methods have been utilized for induction of homoeologous chromosome pairing by manipulating, suppressing or eliminating 5B chromosome activity. Use of 5B-deficient stocks like Langdon 5D(5B) disomic substitution lines favours intergenomic chromosome pairing and leads to effective interspecific and intergeneric gene transfers (Sears 1981; Jauhar et al. 2009). Using this method, the genes for scab resistance have been transferred successfully from diploid wheat grass *L. elongatum* to wheat (Jauhar et al. 2009). The recessive mutant of the homoeologous pairing suppressor *Ph1* can also be used as an effective method for induction of homoeologous pairing (Riley and Chapman 1958; Zhang et al. 2004). Another homoeologous pairing suppressor allele, *Ph2*, with much weaker expression has been located on short arm of 3D chromosome of wheat (Mello-Sampayo and Canas 1973), but it was rarely used to enhance pairing, except by Ceoloni and Donini (1993). The other method that enhances homoeologous pairing is crossing of wheat with *Ae. speltoides* which suppresses the action of the *Ph1* gene due to the presence of the *Ph1* suppressor genes *Su1-Ph1* and *Su2-Ph1* (Dvorak et al. 2006). Chen et al. (1994) successfully transferred Ph-suppressor genes from *Ae. speltoides* into bread wheat line which was used as an efficient inducer of homoeologous pairing since *PhI* genes are dominant and epistatic to the wheat *Ph*. This method has been successfully exercised to transfer leaf rust and stripe rust resistance genes from *Ae. umbellulata* (Chhuneja et al. 2008) and from *Ae. triuncialis* and *Ae. geniculata* (Aghaee-Sarbarzeh et al. 2002) to bread wheat.

1.3.3.2 Induction of Alien Translocations by Ionizing Irradiation

To avoid the linkage drag in alien addition and substitution lines, alien translocation method was utilized for transfer of small segments (Sears 1981). The inherent

drawback of this system is reduced number of chiasmata (1–2) per pair of chromosomes during pairing between donor and elite lines and predominant occurrence of chiasmata in the terminal and subterminal chromosome regions. Genes located in the more proximal regions of the chromosomes will not be included in the translocated segment or will be transferred together with a large alien chromosome segment. However, a technique standardized by Sears allows transfer by making use of X-rays to induce centromeric breaks and consequent spontaneous fusion of alien chromatin with that of wheat (Sears and Gustafson 1993). This technique was used to transfer *Lr9* gene for leaf rust resistance from *Ae. umbellulata* into wheat. However, the breaks induced were random leading to translocations among non-homoeologous chromosomes resulting in the generation of duplications or deficiencies in the progeny. Such genomic modifications are non-compensating and agronomically undesirable.

A method to reduce a whole-arm translocation into small segments was also presented by Chen et al. (2008) in support of wheat × *Dasypyrum villosum* introgressions. A 6VS/6AL translocation line was gamma irradiated and immediately pollinated by wheat giving rise to an impressive number of small terminal and interstitial alien translocations.

1.3.3.3 Gametocidal Induction of Alien Translocations

Another method to transfer small alien segments of rye into wheat is based on the capacity of gametocidal genes to break chromosomes. Gametocidal genes (*Gc*) are a group of selfish genes that induce chromosome breakage in gametes not having them (Tsujimoto 1995), This mechanism prevents the transmission of these gametes and ensures that only gametes containing the *Gc* genes are transmitted. Consequently, the *Gc*-carrying *Aegilops* chromosome is included in the genome of every offspring derived from self-pollination or backcrossing of the wheat—*Aegilops* hybrid. Masoudi-Nejiad and co-workers (2002) exploited the action of gametocidal genes to transfer alien chromosome segments of *Ae. triuncialis* to wheat. Many small segments of 1R were translocated to different wheat chromosomes as a result of chromosome breakage caused by the gametocidal gene located on 3C chromosome.

1.3.3.4 Characterization of Alien Chromatin in Wheat Genome

Development of F_1 generation after hybridization of two distantly related species is not sufficient to conclude successful introgression. Cytological verification appears to be more convincing but may result in misinterpretation due to technical problems in collection of samples, choice of technique used, somatic chromosomal elimination or chimera synthesis and false impression of meiotic data. Classically, the recombination in chromosomes was identified using their morphological markers and behaviour (chiasmata formation) during meiosis (Sears 1981). The introduction of differential staining, particularly Giemsa C-banding, allowed unambiguous chromosome identification of paired homoeologous chromosomes in meiotic metaphase I and characterization of structural changes (Gill et al. 2011). The innovation of in situ

hybridization (ISH) (Gall and Pardue 1969) and application of GISH and FISH to monitor alien introgressions in wheat (Yamamoto and Mukai 1989; Schwarzacher et al. 1989) have opened new frontiers for enhancing precision in introgression of alien chromatin in wheat. Cytogenetic techniques continue to play a pivotal role even in the genomics era by facilitating the physical mapping of molecular markers and expressed sequences (EST) (Chaudhary 2004; Schwarzacher et al. 2011). Further enhancement in the efficiency of FISH by the application of tyramide FISH (Tyr-FISH), 3-D FISH and fibre FISH aided in the diagnostic study of interphase chromosomes up to 5–500 kb. Furthermore, the availability of a wide range of fluorescent labelling dyes and hybridization molecules has enabled the use of multicolour-FISH (M-FISH) (Speicher et al. 1996), spectral karyotyping (SKY) (Schrock et al. 1996) and combined binary ratio labelling (COBRA) for deciphering the intergenomic pairing among more than two genomes of different species. The exploitation of tandem sequence repeats like 28S, 26S and 5.8S rRNA gene subunits (Mukai et al. 1991) and 5S RNA genes (Mukai et al. 1990) as molecular probes in the cereal genome including wheat has opened a plethora of possible gene mapping (Jaberson et al. 2012).

Although, the resolution of molecular cytogenetic techniques has enabled the wheat breeders to reach the gene level, but it cannot replace the utility of molecular techniques at DNA level. The use of DNA hybridization technology and PCR-based amplification or the combination of these two has widened the horizons and deepened the resolution of molecular characterization leading to the more precise alien introgression in wheat. Amplification of nuclear organizer region, 5S rRNA sequence in *R1*-locus and *Ris-1* repetitive element of rye has hastened the detection of 1R substitutions and 1RS translocations in the wheat genome (Van Campenhout et al. 1998). RFLP analysis combined with slot–blot hybridization has proved amenable for detection of 1R rye introgressions in wheat. The construction of SSR maps has facilitated the generation of high-density genetic maps of wheat genome which enabled the identification of key recombination events in breeding populations and fine mapping of genes (Somers et al. 2004). A number of genes governing important agronomic traits including disease resistance, vernalization response, grain quality and abiotic stress tolerance have recently been cloned by map-based cloning (Gupta et al. 2008). Flow sorting and microdissection of chromosomes have been used for detection of morphological as well as numerical changes in the genome, alien chromosomes and segments and are amenable in construction of chromosome-specific BAC libraries (Chalhoub et al. 2004) to facilitate map-based cloning of agronomically important QTLs like Fusarium head blight resistance (Liu et al. 2005). Targeting induced local lesions in genome (TILLING) has emerged as a valuable reverse genetic approach to recognize the function of specific genes and has been extensively used in locating the diversity of *waxy* locus which can further be used in allele mining in elite wheat germplasm (Slade et al. 2005). Comparative genomics further led to the dissection of the genes controlling various quality and adaptability traits including gene(s) controlling pre-harvest sprouting, grain hardness, glume coloration and pubescence and the *Pm43* gene, responsible for resistance against powdery mildew (He et al. 2009). Thus, the availability of molecular tools has not only enabled the plant breeders to identify even minute variability in the genome but also hastened the wheat improvement programmes through marker-assisted selection.

1.4 Achievements of Sexual Alien Gene Transfer

Wild relatives of wheat are important sources of new genes for cultivated wheat. In the past 40 years, numerous desirable genes, including approximately 20 stem rust resistance genes (Qi et al. 2011), have been transferred into common wheat from its wild relatives by developing wheat-alien species chromosome translocation lines through chromosome engineering (Friebe et al. 1996; Gill et al. 2011). Chromosome engineering has also been used sparingly in durum wheat, but the successful transfer of genes for high-molecular-weight glutenins, disease resistance, salt tolerance and kernel texture has been documented. The list of alien chromosomes/genes that have been transferred successfully in wheat till now is depicted in Table 1.1.

Table 1.1 Successful alien gene introgressions in wheat

Gene/allele	Location	Source	Reference
Genes encoding resistance for powdery mildew			
Pm2	5DS	*Ae. tauschii*	Lutz et al. (1995)
Pm4	2AL	*T. monococcum*	Schmolke et al. (2012)
Pm7	4BS4BL-2RL	*S. cereale*	Friebe et al. (1996)
Pm8	1RS-1BL	*S. cereale*	Hsam and Zeller (1997)
Pm12	6BS-6SS-6SL	*Ae. speltoides*	Jia et al. (1996)
Pm13	3BL-3SS-3S	*Ae. longissima*	Ceoloni et al. (1992)
Pm19	7D	*Ae. tauschii*	Lutz et al. (1995)
Pm20	6BS-6RL	*S. cereale*	Friebe et al. (1996)
Pm21	6VS-6AL	*Haynaldia villosa*	Xie et al. (2012a)
Pm26	2BS	*T. dicoccoides*	Rong et al. (2000)
Pm27	6B-6G	*T. timopheevii*	Jarve et al. (2000)
Pm29	7DL	*A. ovate*	Zeller et al. (2002)
Pm29	7D	*Ae. geniculata*	Stoilova and Spetsov (2006)
Pm30	5BS	*T. dicoccoides*	Liu et al. (2002)
Pm31 (MIG)	6AL	*T. dicoccoides*	Xie et al. (2003)
Pm32	1BL	*Ae. speltoides*	Hsam et al. (2003)
Pm33	2BL	*T. carthlicum*	Zhu et al. (2005)
Pm34	5DL	*Ae. tauschii*	Miranda et al. (2006)
Pm35	5DL	*Ae. tauschii*	Miranda et al. (2007)
Pm36	5BL	*T. dicoccoides*	Blanco et al. (2008)
Pm40	7BS	*Elytrigia intermedia*	Luo et al. (2009)
Pm41	3BL	*T. dicoccoides*	Li et al. (2009)
Pm43	2DL	*Th. intermedium*	He et al. (2009)
PmG3M	6BL	*T. dicoccoides*	Xie et al. (2012b)
PmTm4	7BL	*Secale cereale*	Hu et al. (2008)
PmTb7A.1	7A	*T. boeoticum*	Chhuneja et al. (2012)
PmT7A.2	7A	*T. boeoticum*	Chhuneja et al. (2012)
PmG25	5BL	*T. turgidum*	Alam et al. (2013)
Mlm80	7AL	*T. monococcum*	Yao et al. (2007)
mlW172	7AL	*T. dicoccoides*	Ji et al. (2008)

(continued)

Table 1.1 (continued)

Gene/allele	Location	Source	Reference
MlIW170	2BS	*T. turgidum*	Liu et al. (2012)
MlNCD1	7D	*Ae. tauschii*	Maxwell et al. (2012)
Ml5323	2BS	*T. turgidum*	Piarulli et al. (2012)
Genes encoding resistance for stem rust			
Sr26	T6A-6Ae#lL	*Th. ponticum*	Friebe et al. (1996)
Sr31	1BL-1RS	*Secale cereale*	Das et al. (2006)
Sr34	2A	*Ae. comosa*	Xu et al. (2008)
Sr36	2BS	*T. timopheevii*	Friebe et al. (1996)
Sr37	4BL	*T. timopheevii*	Friebe et al. (1996)
Sr39	2BS	*Ae. speltoides*	Mago et al. (2009)
Sr40	2BS	*T. timopheevii*	Xu et al. (2008)
Sr43	7D	*Th. intermedium*	Kim et al. (1993)
Sr44	T7DS-7Ai#lL	*Th. intermedium*	Liu et al. (2013a)
Sr51	3AL	*Ae. searsii*	Liu et al. (2011a)
Sr52	T6AS.6V#3L	*Dasypyrum villosum*	Qi et al. (2011)
Sr53	T5DL.5ML.5MS	*Ae. geniculata*	Liu et al. (2011b)
Sr 54	2D	*Ae. speltoides*	Ghazvini et al. (2013)
Genes encoding resistance for leaf rust			
Lr18	5BS	*T. timopheevii*	Friebe et al. (1996)
Lr19	7DL	*Th. ponticum* and *Th. distichum*	Prins et al. (1996)
Lr28	4AL	*Ae. speltoides*	Naik et al. (1998)
Lr38	lDS-lDL	*Th. intermedium*	Friebe et al. (1996)
Lr39	2DS	*Ae. cylindrica*	Singh et al. (2003)
Lr40	1DS	*Ae. tauschii*	Hiebert et al. (2007)
Lr41	2DS	*Ae. cylindrica*	Singh et al. (2003)
Lr50	2BL	*T. timopheevii*	Brown-Guedira et al. (2003)
Lr54	1D	*Ae. kotschyi*	Marais et al. (2005)
Lr55	1BL	*Elymus trachycaulus*	Friebe et al. (1996)
Lr56	6A	*Ae. sharonensis*	Marais et al. (2006)
Lr57	T5DL-5MS	*Ae. geniculata*	Kuraparthy et al. (2007a)
Lr58	2BL	*Ae. triuncialis*	Kuraparthy et al. (2007b)
LrAC	5DS	*Ae. caudate*	Riar et al. (2012)
Genes encoding resistance for stripe rust			
Yr5	2BL	*Triticum spelta*	Smith et al. (2007)
Yr9	1B.1RS	*Secale cereale*	Mago et al. (2002)
Yr26	1 BS	*T. turgidum*	Ma et al. (2001)
Yr28	4DS	*Ae. tauschii*	Singh et al. (2000)
Yr35	6BS	*T. dicoccoides*	Dadkhodaie et al. (2011)
Yr36 (HTAP)	6BS	*T. dicoccoides*	Uday et al. (2005)
Yr37	2D	*Ae. kotschyi*	Marais et al. (2005)
Yr38	6A	*Ae. sharonensis*	Marais et al. (2010)
Yr40	T5DL-5MS	*Ae. geniculata*	Kuraparthy et al. (2007a)
Yr42	6AL (6L.6S)	*Ae. neglecta*	Marais et al. (2009)
Yr50	4BL	*Th. intermedium*	Liu et al. (2013b)
Yr53	2BL	*T. durum*	Xu et al. (2013)

(continued)

Table 1.1 (continued)

Gene/allele	Location	Source	Reference
YrM8003	7DS	*Secale cereale*	Xu et al. (2010)
YrSn0096	4AL	*Leymus mollis*	Bao et al. (2012)
YrHA	1AL	*Ps. huashanica* keng	Ma et al. (2013)
Gene encoding resistance for head blight			
Fhb3	T7AS.7Lr#1S	*Leymus racemosus*	Qi et al. (2008)
Fhb gene	T7AS#7ES	*Th. elongatum*	Fu et al. (2012)
Genes encoding resistance for barley dwarf virus			
Bdv2	T7DS-7Ai#lL	*Th. intermedium*	Banks et al. (1995)
Bdv3	7D	*Th. intermedium*	Ayala-Navarrete et al. (2009)
Bdv4	2D	*Th. intermedium*	Ayala-Navarrete et al. (2009)
Gene encoding resistance for wheat streak virus			
Wsml	T4DL-4Ai#2S	*Th. intermedium*	Wang (1989)
Gene encoding resistance for curl mite			
Cmc2	T6DL-6Ae#2S	*Th. ponticum*	Whelan and Hart (1988)
Genes encoding resistance for root knot nematodes			
Rkn3	T2AS.2N	*Ae. ventricosa*	Williamson et al. (2013)

1.5 Barriers in Sexual Alien Gene Transfer

The list of successful transfers from alien genetic recourses seems to be heading towards new horizons, but still there are some bottlenecks which prevent the achievement of desired goals. It is not always as easy as it seems to transfer the alien chromatin through wide hybridization as the transfer is largely hampered by a number of factors. From time to time, breeders have found solutions to the obstacles coming into their way, yet there are many issues which are to be resolved before moving ahead. The major barriers for wide hybridization are

- Pre-fertilization barriers
- Post-fertilization barriers
- Barriers to gene expression in progeny of wide hybridization

Pre-fertilization barriers prevent union of gametes which may be due to failure of pollen to reach stigma, improper germination of the pollen and inhibition of growth of the pollen tube in the style. Post-fertilization reproductive barriers lead to abnormalities following fertilization like hybrid inviability or weakness and sterility of plants. After fertilization, various hindrances to proper development of the hybrid embryo may arise that in some cases may result in abortion of the embryo or even formation of a haploid due to elimination of the whole alien chromosome complement. The breeder may use embryo rescue techniques to remove the embryo and culture it on artificial medium. The hybrid sterility may be overcome through chromosome doubling (Claesson et al. 1990). Furthermore, after obtaining fertile plants either through chromosome doubling or embryo rescue, alien gene transfer has

other disadvantages and difficulties, like linkage drag that requires time-consuming backcrosses and simultaneous selection steps and less recombination between the chromosomes in hybrids to obtain desired combinations. In order to have desired recombination between diverse genomes, induction of homoeologous pairing is necessary which needs chromosome manipulation techniques that are very difficult and cumbersome. The chromosomes of many alien species do not recombine with those of the wheat due to the presence of a pairing control gene, *Ph1*. This gene has to be manipulated, suppressed or eliminated so that the chromosomes of wheat and alien species may recombine.

1.6 Biotechnology-Assisted Gene Transfers

1.6.1 Direct Gene Transfer

Alien introgression from related species through sexual hybridization and cytogenetic techniques has contributed to a great extent in wheat improvement endeavours. However, these methods are not sufficient in some cases to achieve the target and have some inherent drawbacks like linkage drag, crossability and species barriers that impede their utilization in wheat improvement programmes. Genetic engineering offers an excellent tool for inserting well-characterized genes from unrelated organisms into plant cells asexually, which on regeneration produce plants with inserted genes integrated in their genome. Moreover, this novel technology allows access to an unlimited gene pool without the problems of sexual incompatibility, species barriers and linkage drag. Any modification can be designed and tailored to achieve desired effects through this approach.

Despite the global importance of genetic engineering, wheat was the last cereal to be genetically transformed due to its recalcitrant nature and genotype dependence of foreign DNA transfer by *Agrobacterium* (Bhalla et al. 2006). Wheat has been successfully transformed using micro-projectile bombardment (Vasil 2007) and *Agrobacterium*-mediated transformation methods (Wu et al. 2003; He et al. 2010). Both these methods involve delivery of the transgene to callus tissues followed by selection of transformed cells and regeneration of plantlets carrying the gene of interest. Transgenic wheat facilitates the improvement of wheat quality; reduces the enormous yield losses due to weeds, pests, pathogens, heat, frost and drought and contributes to increased yields under optimal conditions (Yu et al. 2010).

1.6.1.1 Achievements of Direct Alien Gene Transfer

Following the production of the first transgenic wheat plants by Vasil et al. (1992), many agronomically important and useful genes, such as those for resistance to biotic (herbicides, pests, pathogens, etc.) and abiotic (drought, soil salinity, heat or cold, etc.) stresses, improved bread-making and nutritional qualities and increased yield,

have been introduced into wheat (Patnaik and Khurana 2001; Saad et al. 2013). The various traits that have been improved in wheat through transformation by various studies are listed in Table 1.2.

Recently, Saad et al. (2013) introduced a rice stress-responsive transcription factor encoded by the rice *NAC1* gene (*SNAC1*) that plays an important role in drought stress tolerance into an elite Chinese wheat variety "Yangmai12" under the control of a maize ubiquitin promoter. Plants expressing *SNAC1* displayed significantly enhanced tolerance to drought and salinity in multiple generations and contained higher levels of water and chlorophyll in their leaves as compared to wild type.

Table 1.2 Transgenes for various traits transferred into wheat

Gene	Source	Function	Reference
HMW-GS	*Thinopyrum intermedium*	Improvement in gluten protein	Li et al. (2013)
TiMYB2R-1	*Thinopyrum intermedium*	Resistance to take-all disease	Liu et al. (2013c)
BLF gene	Bovines	Resistance to head blight	Han et al. (2012)
RsAFP2	Radish	Resistance to *Fusarium* and *Rhizoctonia*	Li et al. (2011)
HVA1 gene	Barley	Drought tolerance	Chauhan and Khurana (2011)
betA gene	*Escherichia coli*	Drought tolerance	He et al. (2011)
NIa gene	WSM virus	Immunity to wheat streak mosaic virus	Fahim et al. (2010)
Ama1	*Amaranthus hypochondriacus*	Improve nutritional quality	Tama's et al. (2009)
GhDREB gene	Cotton	Drought, salt, freeze stress	Gao et al. (2009)
P5CS gene	*Vigna aconitifolia*	Drought tolerance	Vendruscolo et al. (2007)
NPR1	*Arabidopsis thaliana*	Resistance to *Fusarium* head blight	Makander et al. (2006)
GmDREB	Soybean	Drought and salt	Gao et al. (2005)
PIN2	Potato	Cereal cyst nematode infestation	Vishnudasan et al. (2005)
DREB1A gene	*Arabidopsis thaliana*	Drought tolerance	Pellegrineschi et al. (2004)
mtlD gene	*Escherichia coli*	Drought and salinity	Abebe et al. (2003)
FsTRI101	*Fusarium sporotrichioides*	Protection against the spread of *F. graminearum*	Okubara et al. (2002)
P5CS	*Vigna aconitifolia*	Increased tolerance to salt	Sawahel and Hassan (2002)
Coat protein gene (CP)	Wheat streak mosaic virus	Resistance to wheat streak mosaic virus	Sharp et al. (2002)
CP gene	Wheat streak mosaic virus	Resistance to wheat streak mosaic virus	Sivamani et al. (2002)
Pmi, gene	*Escherichia coli*	Metabolize mannose, into fructose	Wright et al. (2001)

(continued)

Table 1.2 (continued)

Gene	Source	Function	Reference
tlp antisense gene	Barely	Resistant to *Alternaria triticina* pathogen	Pellegrineschi et al. (2001)
Rnc70	Barley stripe mosaic virus	Resistance to stripe mosaic virus	Zhang et al. (2001)
PhyA gene	*Aspergillus niger*	Accumulation of phytage in transgenic seeds	Brinch-Pedersen et al. (2000)
KP4	*Ustilago maydis* infested virus	Resistance against stinking smut	Clausen et al. (2000)
RIP gene	Barley	Only moderately or not at all against the fungal pathogen *Erysiphe graminis*	Bieri et al. (2000)
Viral replicase (Nib)	Wheat streak mosaic virus	Resistance to wheat streak mosaic virus	Sivamani et al. (2000b)
HVA1 gene	Barley	Drought tolerance	Sivamani et al. (2000a)
Stilbene synthase (Sts)	*H. vulgare* L.	Production of phytoalexin resveratrol	Fettig and Hess (1999)
Chi 2	Barely	Increased resistance to *Erysiphe graminis*	Bliffeld et al. (1999)
Ac gene	Maize	Plants expressing the transposase	Takumi et al. (1999)
Stilbene synthase (Vst1)	*Vitis vinifera*	Production of phytoalexin resveratrol	Leckband and Lörz (1998)
Bar gene	*Streptomyces hygroscopicus*	Resistant to broad-spectrum herbicide	Vasil et al. (1992)

A promising approach to breed wheat cultivars with improved tolerance for pre-harvest sprouting through a genetic engineering process has been recently revealed by incorporating the viviparous-1 (*Vp1*) gene from maize which encodes a transcription factor involved in the abscisic acid (ABA) synthesis into elite wheat variety by *Agrobacterium tumefaciens* (Huang et al. 2012).

1.6.1.2 Challenges and Limitations

To accelerate the process of commercially using genetically modified (GM) wheat, new transgenic methodologies should be developed as the current methods are laborious and time consuming. Technical hurdles also remain in the establishment of a high-throughput wheat transformation platform, development of elite wheat varieties without deleterious selection marker genes and introduction of a minimal backbone or no bacterial DNA sequences, enacting responsible, rigorous and feasible regulatory systems and issues involving intellectual property rights and social and market acceptance of GM wheat. Thus, genetic transformation should be used only in those instances, where alien gene transfers are not possible in a timely and useful manner through conventional means.

1.6.2 Doubled-Haploid Production

In vitro haploid induction followed by chromosome doubling offers an efficient means for instant fixation of alien introgressions by attaining complete homozygosity in a single generation. Besides this, the unique characteristics and complete homozygous nature of doubled haploids (DHs) make them very useful for early release of cultivars, germplasm development, understanding the inheritance mechanism of complex quantitative traits, mapping quantitative trait loci (QTLs) associated with multigenic traits, genomics studies, gene identification, whole-genome mapping and accelerated production of transgenic plants. The commonly used methods of DH production in wheat are anther culture, wheat × maize and wheat × *Imperata cylindrica*. The androgenesis-mediated haploid induction methods are not generally used in wheat improvement programmes due to genotype specificity and poor response of wheat varieties to anther culture. The wheat × maize system, though a genotype non-specific and more efficient approach of haploid induction in wheat, has failed to produce haploids in wide hybrids like triticale × wheat and wheat × rye (Kishore et al. 2011). Wheat × *Imperata cylindrica-mediated* chromosome elimination approach of doubled-haploidy breeding has been identified as the most efficient alternative for haploid induction in wheat (Chaudhary 2008a, 2008b, 2010, 2011, 2013a, 2013b; Chaudhary et al. 2002, 2005, 2013a, 2013b; Komeda et al. 2007; Tayeng et al. 2012; Rather et al. 2013). Similar to wheat × maize system, *I. cylindrica*-mediated system is also genotype non-specific and insensitive to crossability inhibitor genes. This novel system is capable of inducing haploids in wheat × rye and triticale × wheat derivatives, where maize was not successful (Kishore et al. 2011; Pratap and Chaudhary 2007, 2012; Pratap et al. 2005, 2006).

The DHs can be used for transformation of desirable gene, and thus stable transgenic plants having the desired gene are produced (Foster et al. 2010). The advantage of haploids in transformation process is that once a transgene is transferred, transgenic plants can be used straight away to produce homozygous lines. Otherwise, a lot of labour and time of at least two generations are required to separate homozygous and heterozygous lines (Patnaik and Khurana 2001). The transgene can be transferred by bombarding the gene on haploid embryos or by growing *Agrobacterium* and haploid embryos together. Transformation using *Agrobacterium* has several advantages over direct gene transfer by gene gun like high success rate, possibility of transferring longer DNA fragments and copying or cloning of genes (Khurana et al. 2011). DH transgenic plants were tested for drought tolerance, and it was found that the gene "*HVA1*" was transferred and expressed successfully (Chauhan and Khurana 2011). This clearly indicates that DH technology can be successfully used for production of stable transgenic plants. Thus, this system plays a vital role in crop improvement programmes.

1.7 Conclusion

In near future of climate change, we need higher yielding and more nutritious wheat varieties that are resistant to a wide range of biotic and abiotic stresses. Thus, genetic improvement of wheat in a sustainable way is of key concern for wheat breeders. For the genetic approach and breeding to be successful, a continuing infusion of new genetic diversity represents a necessary request in the era of global climate change. Variability for various traits is limited or even exhausted within primary wheat gene pool. Secondary and even tertiary gene pools represent a wide and less exploited reservoir of desirable alien genes that can be incorporated into cultivated genotypes of wheat through appropriate methods like wide hybridization and genetic transformation. Although wide hybridization has been practiced globally and frequently for alien gene transfer into wheat, still its use as the frontline approach is limited due to unavoidable reasons like sexual incompatibility, F_1 sterility and hybrid inviability. Gene transfer through chromosome engineering/chromosome manipulation is an already available tool that may provide high-yielding wheat elite lines with tolerance to abiotic and biotic stresses by introgressing genes from uncultivable gene pool and will diversify the primary gene pool in a sustainable way. However, the inherent problems of sexual incompatibility, crossability, fertility, linkage drag and recombination of genes in case of alien gene introgression must be addressed and overcome by utilizing appropriate advanced procedures coupled with the use of biotechnological tools. Transgenic wheat plants should be produced and used only when they provide substantial improvements and advantages over conventional, mutation and marker-assisted breeding and selection.

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Chapter 2
Maize

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Abstract During the domestication process of cultivated crop species, the variability has decreased considerably rendering them more susceptible towards vagaries of environment and biotic stresses. Alien genes from wild relatives are not only the source of imparting new genetic variability but are also storehouses of novel traits which are linked with wider adaptability towards biotic and abiotic stresses. Transfer of alien genes from wild species to maize has been undertaken for a number of traits of agronomic value and for imparting resistance to stresses using both conventional and biotechnological tools. This chapter covers the problems encountered in transfer of alien chromatin into maize and the tools to overcome those problems including direct gene transfer of alien DNA beyond taxonomical boundaries. It also presents various achievements in introgression of alien genes via various methods and also the impact of such introgressions on maize improvement.

Keywords Alien gene transfer • Achievements • Biotechnology • Direct gene transfer • Impacts • *Teosinte* • *Trypsacum* • Somatic hybridization

2.1 Introduction

Maize (*Zea mays* L.), also known as corn, is the third most important cereal after wheat and rice that is grown and consumed in the form of food, animal feed and in several industrial processes. Maize, the queen of cereals, is one of the oldest domesticated plants dating back to as far as 7,000 years ago in Central Mexico by

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Mesoamerican natives. The crop seems to develop as a result of gradual selection upon primitive annual teosinte (*Zea mexicana*), an ancient grass found in Mexico and Guatemala, although opinions vary as to whether maize is a domesticated version of teosinte (Galinat 1988). Maize is being cultivated in the tropical, subtropical and temperate climatic regions of Asia, Africa, Europe and North and South America. Currently, the area under this crop is nearly 162 m ha, out of which, nearly 100 million ha is covered by 125 developing countries with global production reaching a mark of 845 m tonnes with global productivity of 5.21 tonnes/ha, 67 % coming from low- and lower middle-income countries (Anonymous 2012). In terms of international trade, although maize accounts for only 12 % of worlds cereal production, it represents over one-third of cereal trade. About 30 % of world's production is used for direct human consumption and as an industrial input, while 70 % is used as an animal feed (Turrent and Serratos 2004).

Maize along with wheat and rice provides at least 30 % of the food calories to more than 4.5 billion people in 94 developing countries where one-third of children are malnourished (Hoisington et al. 1999; Von Braun et al. 2010). By 2050, the demand for maize in the developing world will be almost double the current demand (Rosegrant et al. 2008), and with the current scenario of production which is lagging far behind, estimates are not quite optimistic for the poor and marginal farm families. Under the changing climate scenario, there is a further threat to maize production, and the low- and middle-income countries will be the most affected by climate change. Spatial analyses in recent years have consistently predicted an average of 10 % or even more decline in maize yields by 2050 for sub-Saharan Africa and Latin America (Thornton et al. 2010; Lobell et al. 2011). In addition to abiotic stresses, increasing maize production is threatened by as much as about 110 diseases on a global basis caused by fungi, bacteria and viruses and 130 insect-pests.

2.2 Maize Improvement

Although a rapid boost in maize production was achieved as a result of using single, double and three-way crosses, the hybrid technology has also posed a challenge on meeting the target growth in maize production due to narrowing down of genetic variability. Concerns over the lack of genetic diversity in maize used for production go beyond the realms of academic argument and theory. The devastating 1970 epidemic of southern corn leaf blight (caused by the fungal pathogen *Bipolaris maydis* race T) was due to the widespread deployment of genetically uniform varieties, all containing T-cytoplasm. In addition to increased susceptibility to diseases and pests, low diversity levels do not bode well for yield plateaus lurking on the horizon. Exotic maize germplasm contains significant variation for many quality traits that have remained untapped (Hoisington et al. 1999). Also, the threat of genetic vulnerability can be overcome by use of wild and distant relatives of maize to broaden the genetic base of maize germplasm and also to introgress resistance against biotic and abiotic stresses.

Genetic variability is a basic element necessary to any plant breeding programme. If genetic variability is not present in the breeder's populations, selection will not be.

Plant breeders devote considerable effort and time to the development of genetically variable populations for future selection. The mechanisms used for creating genetic variability include hybridization of adapted material, mutagenic agents and introduction of germplasm from other sources (Hallauer and Sears 1972). Introduction of exotic maize germplasm to improve and broaden local maize genetic base is a widely used method across the world especially for agronomically important traits (Albrecht and Dudley 1987; Vasal et al. 1992a, b; Ron Parra and Hallauer 1997; Goodman 1999; Abadassi and Hervé 2000; Li et al. 2001; Ho et al. 2002; Chen et al. 2010). Moreover, when the variability for certain traits is not available in the exotic germplasm, distant relatives are used to introgress alien genes into genetic background.

2.3 Sources of Alien Genes

Maize belongs to the family Poaceae and tribe Maydeae which comprises seven genera, viz. Coix ($2n=10$ or 20), Chionachne ($2n=20$), Sclerachne ($2n=20$), Trilobachne ($2n=20$) and Polytoca ($2n=20$) (Old World groups) and *Zea* and *Tripsacum* (New World groups). The genus *Zea* consists of four species of which only *Z. mays* L. ($2n=20$) is economically important. The other *Zea* sp., referred to as teosintes, are largely wild grasses native to Mexico and Central America (Doebley 1990). Species belonging to both *Zea* and *Tripsacum* serve as important sources of alien genes for introgressing desirable traits into genetic background of bread wheat (Table 2.1).

2.3.1 Teosintes

Teosintes are wild grasses native to Mexico and Central America with limited distribution. Among teosintes, the nearest teosinte relative to *Z. mays* is *Z. mays* sp. *mexicana* (Schrader) Iltis ($2n=20$), distributed across central highlands of Mexico. The other teosintes include perennial teosintes, viz. *Z. diploperennis* ($2n=20$) and *Z. perennis* ($2n=40$), distributed in Jalisco, Mexico. The annual teosintes include *Zea luxurians* from southeastern Guatemala, *Zea mays* ssp. *parviglumis* of southern and western Mexico and *Zea mays* ssp. *huehuetenangensis* from the western highlands of Guatemala (Reeves and Mangelsdorf 1942; Hitchcock 1951; Iltis et al. 1979; Iltis and Doebley 1980; Doebley 1990; Watson and Dallwitz 1992).

2.3.2 Tripsacum

The genus *Tripsacum* is comprised of about 12 perennial and warm season species that are mostly native to Mexico and Guatemala but are widely distributed throughout warm regions in the USA and South America, with some species present in Asia

Table 2.1 Classification of the genus *Zea* within the tribe Maydeae and the genus *Tripsacum*

Species	Chromosome number (2*n*)	Common name
Zea mays subsp. *mays* (L.) Iltis	20	Maize
Zea mays subsp. *mexicana* (Schrader) Iltis	20	Teosinte
Zea mays subsp. *parviglumis* Iltis and Doebley	20	Teosinte
Zea mays subsp. *huehuetenangensis* Doebley		Teosinte
Zea diploperennis Iltis, Doebley and Guzman	20	Perennial teosinte
Zea luxurians (Durieu) Bird	20	Teosinte
Zea nicaraguensis	20	–
Zea perennis (Hitchc.) Reeves and Mangelsdorf	40	Tetraploid teosinte
Genus *Tripsacum*		
T. andersonii	64	–
T. australe	36	–
T. bravum	*3*6, 72	–
T. cundinamarce	36	–
T. dactyloides	72	Eastern gamma grass
T. floridanum	36	–
T. intermedium	72	–
T. manisuroides	72	–
T. latifolium	36	–
T. pereuvianum	72, 90, 108	–
T. zopilotense	36, 72	–
T. jalapense	72	–
T. lanceolatum	72	
T. laxum	36?	
T. maizar	36, 72	
T. pilosum	72	

and Southeast Asia. Species of economic importance to agriculture in the genus are *Tripsacum dactyloides* (L.) (Eastern gama grass), *T. laxum* Scrib and Merr. Other species include *T. andersonii*, *T. latifolium*, *T. lanceolatum*, *T. floridanum* and *T. manisuroides* (deWet and Harlan 1972; deWet et al. 1983; Talbert et al. 1990; Watson and Dallwitz 1992).

2.3.3 Coix and Other Asiatic Genera

The Asiatic genera of the Maydeae tribe are native to an area extending from India to Southeast Asia and the Polynesian islands to Australia. They include Coix L. (2*n*=10, 20 and 40), Sclerachne R. Br. (2*n*=20), Polytoca R. Br. (2*n*=20 and 40), Chionachne R. Br. (2*n*=20) and Trilobachne Schenk and Henrard (2*n*=20). Coix sp. is the most familiar genera and includes several species. The species *Coix lacryma-jobi* Linn. (Job's Tears) (2*n*=20) is native to Southeast Asia, Africa and warmer parts of the

Mediterranean and exists in the wild as well as cultivated races. Chionachne includes several species native to Southeast Asia. The species *C. semiteres* is cultivated as a fodder crop. Polytoca includes a few species, none of which however are commonly cultivated. One species has been described for Trilobachne and is not known to be cultivated. Both genera are native to Southeast Asia (Watson and Dallwitz 1992).

2.4 Crossability Potential and Barriers for Alien Gene Transfer

The success of a breeding programme depends upon the production of fertile hybrids especially while dealing with interspecific or intergeneric hybridization. Maize exhibits great sexual compatibility with all annual teosintes to produce fertile hybrids except for the tetraploid *Z. perennis* (Wilkes 1977). However, maize-teosinte hybrids exhibit low fitness and have little impact on gene introgression in subsequent generations (Galinat 1988). The tendency to form natural hybrids differs among teosintes where *Z. luxurians* rarely hybridizes with maize while *Zea mays* sp. *mexicana* frequently forms hybrids (Wilkes 1977). Molecular data confirms that gene flow occurs between Maize and teosintes and suggests that introgression of maize and teosintes occurs in both directions but at low levels (Doebley 1990). Intergeneric crosses are even more difficult with species belonging to genus *Tripsacum*. Nevertheless, *T. dactyloides*, *T. floridanum*, *T. lanceolatum* and *T. pilosum* have been successfully hand crossed with maize to form hybrids. These hybrids have a high degree of sterility and are generally unstable, yet the crossability and stability of *Tripsacum* sp. is genotype dependent (Kindiger and Beckett 1992). Infertility among maize × *Tripsacum* crosses is common because of differences in chromosome number and lack of pairing between chromosomes (Eubanks 1997).

No reports are available regarding the crossability of Asiatic genera with maize; however, genetic and chromosomal studies indicate that the Asiatic genera are very distinct from both maize and teosintes. Nevertheless, the similarity in chromosome number suggests that there may be a potential for crossing to occur between maize and the Asiatic genera (Katiyar and Sachan 1992). Maize readily crosses with hexaploid wheat (*Triticum aestivum*) with high frequencies of fertilization and embryo formation; however, maize chromosomes are eliminated from the genome during the initial stages of meiosis and result in haploid wheat embryos (Laurie and Bennett 1986; Chaudhary et al. 2002; Singh et al. 2004; Sharma et al. 2005). Similarly, maize has also been reported to cross readily with triticale and triticale × wheat derivatives (Pratap et al. 2005, 2006). There have been unsubstantiated reports of hybridization between maize and sugar cane (*Saccaharum* sp.).

The low success of above-mentioned interspecific or intergeneric hybrids is result of cross incompatibility that may arise due to many factors which may be classified into (a) pre-fertilization and (b) post-fertilization barriers (Gutierrez-Marcos et al. 2003).

2.4.1 Pre-fertilization Barriers

2.4.1.1 Difference in Floral Structures and Asynchrony in Flowering

The floral structure and synchrony in flowering time, a prerequisite for any hybridization programme, impose a barrier only during natural fertilization but are easily eliminated under planned hybridization programmes (Kiesselbach 1999; Lausser et al. 2010).

2.4.1.2 Pollen-Style Incompatibility

Partial and complete gametophytic incompatibility has been documented among cultivated maize, landraces and teosinte. The former is demonstrated by differential pollen growth and a skewed recovery of alleles linked to incompatibility genes (Kermicle and Allen 1990). Later Duan et al. (2008) observed that germinating pollen of *Coix lacryma-jobi* L., which elongated till the end of maize pistil, indicated strict reproductive isolation due to pollen-style incompatibility. The pollen-style incompatibility takes place at various levels, viz. competition on the receptive trichomes, inside the transmitting tracts, and elimination of the late-entering tubes at the stigma abscission zone and at a constricted zone of the transmitting tract in the upper ovary wall (Heslop-Harrison et al. 1984).

2.4.2 Post-Fertilization Barriers

2.4.2.1 Abortion of Embryo

The incompatibility in the embryo sac was reported in maize × *Tripsacum* L. and maize × *C. lacryma-jobi* L. var. *frumentacea* by Duan et al. (2008). Partial embryo sac cross incompatibility have also been reported in maize × *Z. mays* ssp. *parviglumis* interspecific crosses.

2.4.2.2 Endosperm Failure

Cooper and Brink (1942) were among the first to ascribe the major role of endosperm in the failure of seed production after interspecific hybridization. Histological studies on hybrid maize showed abnormalities of endosperm development at very early stages ranging from defects at the chalazal pole (Cooper and Brink 1940) to abnormal free nuclear division rates (Brink and Cooper 1940).

2.4.2.3 Hybrid Sterility

The wide hybrids may be sterile due to production of abnormal male and female gametes. Anomalies in the development of the microspore apparently occur because of the failure of chromosomes to congregate at the metaphase plate, development of a tripolar spindle and failure of cytokinesis at the first and second meiotic divisions (Kindiger 1993). The maize-*Tripasacum* hybrids generally have 28 chromosomes (10 from maize and 18 from *Tripsacum*) and exhibit pollen sterility with limited female fertility (Mangelsdorf and Reeves 1939; Newell and de Wet 1973; Mangelsdorf 1974).

2.5 Achievements in Alien Gene Transfer

2.5.1 Utilization of Sexual Hybridization

The wild relatives of any crop species have long been recognized by plant breeders and geneticists as sources of important genes, viz. agronomically desirable traits, resistance to diseases and pests and tolerance to abiotic stresses. Maize has high genetic plasticity and hence is a good candidate for interspecific or intergeneric hybridization (Ramirez 1997). Genetic improvement of crops has traditionally been achieved through sexual hybridization between related or distant species, which has resulted in numerous cultivars with high yields and superior agronomic performance (Prescott-Allen and Prescott-Allen 1986). The desired introgressions from interspecific crosses in maize were not of major concern since fertile hybrids with intermediate phenotype could be recovered easily even as a result of natural hybridization (Ellstrand et al. 2007). The significance of interspecific hybridizations apart from introgression of desirable traits lies in the development of modifier complexes and maintenance of heterozygosity (Mangelsdorf 1952).

2.5.1.1 Transfer of Traits of Agronomic Importance

The traits of agronomic importance are mostly governed by polygenes, and the role of alien germplasm to improve quantitative traits is less reported. The possible reason for this is limitation in introgressing large number of loci responsible for expression of a quantitative trait into the target host (dela Vina et al. 1995). In maize, alien introgression has been accomplished for improvement of agronomic traits using sexual hybridization. Cohen and Gallinat (1984) suggested improvement of maize inbreds with respect to quantitative traits like yield via introgression of alien chromatin segments both from teosintes and *Tripsacum*. The introgression of genetic segments from teosinte was accomplished by selection for modifications of the

female spike during the backcross generations and reported to positively affect quantitatively inherited traits. The *Tripsacum* chromosome was found to increase the combining ability of maize line in which it was incorporated. This segment of alien germplasm, homologous to maize chromosome, contributes increased yield, combining ability and affecting other quantitative traits hence adding new dimensions to the utility of *Tripsacum* germplasm. Sidorov and Shulakov (1962) developed hybrids between maize and teosinte that were having superior silage-making ability. Pásztor and Borsos (1990) reported that maize × teosinte (*Z. mays* ssp. *mexicana*) produced F_1s with profuse tillering and F_2s with teosinte characteristics having more number of tillers, high green matter production and better nutritional quality with respect to lysine, aspartic acid and other amino acids. *Z. mays* ssp. *mexicana* has many important traits, viz. good vigour, high protein in kernel and tillering. Wang et al. (2008) crossed maize with *Z. mays* ssp. *Mexicana* and reported that 54.6 % of the hybrids had higher yield than the superior maize hybrid checks. The advanced backcross generations exhibited improved characters like large number of tillers, increased height, increased 100 seed weight and resistance to lodging. Many researchers use such traits of agronomic importance to confirm the hybridity of plants obtained by crossing maize with its wild relatives (Tang et al. 2004).

2.5.1.2 Transfer of Disease Resistance

Plant breeders have been exploiting wild relatives for introgressing resistance against biotic stresses from over a century. Over 80 % of the beneficial traits conferred by wild relatives involve pest and disease resistance. Findley et al. (1982) introgressed resistance against maize chlorotic dwarf virus (MCDV) into maize from *Zea diploperennis*. The hybrid between maize and *Zea diploperennis* exhibited sterility hence backcross generations were generated which revealed resistance to MCDV. Another teosinte was used to confer resistance against downy mildew in maize. The introgression of resistant genes from *Z. mays* ssp. *Mexicana*, *Z. diploperennis* and *Z. perennis* into maize was carried out by Ramirez (1997) using sexual hybridization. Disease resistance was also imparted from *Z. mays* ssp. *Mexicana* to maize for many diseases, viz. maize stalk rot, maize rough dwarf disease and MCDV. Introgression of resistance against *Fusarium* was reported in F_1 and F_2 generations of crosses between maize and *Z. mays* ssp. *mexicana* (Pásztor and Borsos 1990). Similarly, Bergquist (1979) reported introgression of resistance from distant relatives, viz. *T. dactyloides,* where sexual mating is difficult, against *Colletotrichum graminicola*, *Helminthosporium turcicum*, *H. maydis*, *Erwinia stewartii* and *Puccinia sorghi* by backcrossing into various maize genotypes. In BC_5–BC_{10} generations, resistance to each of the pathogens appeared to be dominant; however, a gradual breakdown of qualitative traits, including resistance, occurred in later generations. Later, Bergquist (1981) successfully transferred a dominant gene Rp^{Td} conferring resistance against rust pathogen of corn *Puccinia sorghi*, from *T. dactyloides*. Similarly, *T. floridanum* was used to introgress resistance gene *Ht* into the genetic background of maize (Hooker and Perkins 1980). Zhou and his associates (1997) conducted the distant

hybridization involving maize × teosinte (*Z. diploperennis* L.) in order to introduce novel genetic variability. They reported fourteen inbred lines resistant to diseases, insects and environmental stress after eighth-generation selfing and selection. Topcrossing of these 14 lines with normal testers produced 1,000 hybrids which showed strong heterosis. On the basis of success of maize × teosinte (*Z. diploperennis*) crosses for introgression of desirable traits, *Z. diploperennis* was suggested as one of the potential sources for widening germplasm pool of maize and to overcome the static situation of maize production in China. Likewise, the alloplasmic inbred lines derived from maize × *Zea diploperennis* interspecific hybrids were reported to exhibit resistance against *H. turcium* and *H. maydis* (Wei et al. 2003).

2.5.1.3 Transfer of Insect Resistance

Insect-pests cause huge yield losses by inducing direct damage to plants and by rendering the grains unfit for human and animal consumption. The major insect-pests of corn are stem and cob borers, rootworms and aphids which are generally polyphagus and damage almost all corn varieties. The wild relatives of maize, viz. *Z. mays* ssp. *mexicana*, *Z. mays* ssp. *diploperennis* and *Z. mays* ssp. *perennis,* have resistance against a number of insect-pests, and these species were used to impart resistance against Asiatic corn borer (Ramirez 1997). Pásztor and Borsos (1990) reported certain degree of resistance in the maize × *Z. mays* ssp. *mexicana* hybrids for corn borer (*Ostrinia nubilalis*). *T. dactyloides* exhibits resistance to corn rootworms via non-preferences and or antibiosis as reported by Branson (1971), Moellenbeck et al. (1995) and Eubanks (2001). Eubanks (1997, 2001, 2002) crossed *Tripsacum* with diploid perennial teosinte and produced viable recombinants that were cross-fertile with maize. This allowed the incorporation of *Tripsacum* genetic material into corn and development of experimental lines, some of which exhibited rootworm resistance, as evidenced in insect bioassays and field root damage ratings (Eubanks 2002; Eubanks 2006). Similarly, Prischmann et al. (2009) produced *Tripsacum*-introgressed maize germplasm in breeding programmes to enhance plant resistance or tolerance to corn rootworms.

2.5.1.4 Resistance Against Parasitic Weed

The parasitic weed *Striga* threatens cereal grain production in tropical and subtropical regions of Asia and Africa. The slow pace of development and deployment of *Striga*-resistant cultivars is mostly attributable to paucity of sources of resistance, complex genetics of resistance and scant knowledge about specific mechanisms associated with expression of resistance in maize to the parasite. Sources of *Striga* resistance in maize have been scarce, perhaps because early evolution and adaptation of the maize crop took place in the absence of this parasite. There have been a few reports which indicated the presence of resistance to *Striga* in wild relatives of maize. Lane et al. (1997) reported that some plants among the wild progenitor of

maize, *Z. diploperennis*, restricted parasite penetration of its roots and impaired the development and survival of parasites. Introgression of resistance genes from *Z. diploperennis* into genetic background of maize against *Striga* has been reported independently by Amusan et al. (2008) and Yallou et al. (2009). Gutierrez-Marcos et al. (2003) reported that *Striga* failed to develop on another wild relative of maize, *T. dactyloides*; however, no post-attachment resistance to *Striga* has been found in cultivars of maize (Oswald and Ransom 2004).

2.5.1.5 Transfer of Abiotic Stress Resistance

Tolerance to Flooding

Flooding damage to maize is highly dependent on the developmental stage of plant, the length of the flooding period and the soil-air temperatures. Maize is affected most by flooding in the early stages of growth and hence is a major concern for maize growers due to huge yield losses and limited availability of flooding-tolerant lines. Although a few maize lines were reported to form adventitious roots at the soil surface during experimental flooding conditions (Mano and Omori 2007), teosintes obtained from regions that are known to receive frequent rainfall may provide a superior genetic resource for the development of flooding-tolerant maize. The teosintes, viz. *Z. nicaraguensis* (Bird 2000; Iltis and Benz 2000), *Z. luxurians* and *Z. mays* ssp. *huehuetenangensis* (Mano et al. 2005a), have been observed to exhibit a higher capacity for adventitious root formation than some maize inbreds. *Z. mays* ssp. *huehuetenangensis* seedlings were observed to exhibit a high adaptability to flooding by developing adventitious roots above the soil surface (Mano and Omori 2007). As a consequence, the adventitious roots of this teosinte can obtain oxygen, and this characteristic may play an important role in its adaptation to flooding conditions. Similarly Z. *nicaraguensis* and *Z. luxurians* were reported to develop well-formed aerenchyma in adult plants (Ray et al. 1999) hence imparting tolerance to flooding conditions.

Salt Tolerance

Since corn has been one of the most sensitive crops to soil salinity and the growth of the crop is highly affected, techniques such as remote hybridization and in vitro selection have been extensively used to accelerate breeding processes with respect to this trait. *T. dactyloides* was suggested as source to resistance against salt stress by Pesqueira et al. (2003) on the basis of their evaluation of hybrids between *Z. mays* ssp. *mays* and *T. dactyloides*. The organogenic calli, induced from immature maize × *Tripsacum* hybrid embryos, were exposed to different NaCl concentrations, and the survival and regeneration percentage was calculated. Plants of the hybrid, obtained from the organogenic calli, were exposed to NaCl concentrations considered harmful for normal growth of maize. The shoot dry weights of plants exposed

to 250 mM NaCl did not show significant differences with respect to the control ones. Although sodium content in shoots was incremented up to two- to fivefold, yet it was not toxic for this material (Pesqueira et al. 2006).

2.5.1.6 Transfer of Apomixis

Apomictic reproduction defines an asexual process that substitutes for sexual reproduction in many species of the family Gramineae. It has been suggested that the development of apomictically reproducing forms provides a major contribution towards food security since it helps in fixation of heterotic, and this effect can be exploited without the need of producing hybrid seed every year. Petrov (1984) advocated the possibility of transferring genes conferring apomixes in *T. dactyloides* L. to maize for the development of true breeding hybrids. The diploid *Tripsacum* reproduce sexually, whereas triploids and tetraploids ($2n=4x=72$) reproduce as facultative or near obligate apomicts. Savidan and Berthaud (1994) reviewed the potential of maize × *Tripsacum* crosses for the transfer of alien genes controlling the diplosporous apomictic mode of reproduction. Likewise, Leblanc et al. (1995) carried out bulk-segregant analysis of F_1 population of maize × *Tripsacum* to identify molecular markers linked to diplospory in *T. dactyloides*. On the basis of maize RFLP probes, three restriction fragments co-segregating with diplospory were identified in one maize-*T. dactyloides* F_1 population that segregated 1:1 for the mode of reproduction. The markers were also found to be linked in the maize RFLP map, on the distal end of the long arm of chromosome number 6. These results support a simple inheritance of diplospory in *Tripsacum*. Later efforts were made to transfer apomixis from diplosporous tetraploid *Tripsacum* into maize through conventional backcrossing. The polyhaploid hybrid plants were totally male sterile, whereas the apomictically produced seeds were viable. Apomictic reproduction in such polyhaploids revealed diploid-like chromosomal complement, confirming diplosporous apomixes. It was also suggested from this investigation that diplosporous apomixes and polyploidy are not totally linked and diploid crops such as maize can also reproduce apomictically (Leblanc et al. 1996).

2.5.1.7 Genetic Analysis

The alien introgressions form wild relatives are not only transfer desirable genes and genetic variability into the cultivated backgrounds but have also proved to be highly useful in revealing the genetic mechanisms at molecular level. The genus *Zea* has been reported as one of the most complex genera, and the direct progenitor of maize is still unknown. To unravel this mystery, the interspecific and intergeneric crosses have been utilized by a number of researchers to understand the events that took place during evolution of *Z. mays*. Recently developed novel molecular cytogenetic tools have been more efficiently used to study the genomic affinities and establish relationship among different species (González et al. 2006).

Other than investigation regarding evolution and species relationships, the wild relatives also helped in dissection of complex traits. Doebley and Stec (1993) investigated two populations derived from interspecific hybrids between maize and two teosintes (*Z. mays* ssp. *parviglumis* and *Z. mays* ssp. *mexicana*) by employing molecular marker loci to map quantitative trait loci (QTLs). Studying these populations, they suggested that in both the populations a relatively small number of loci with large effects were involved in the early evolution of the key traits that distinguish maize and teosinte and are not a specific feature of crop evolution but rather a common phenomenon in plant evolution whenever a species invades a new niche with reduced competition. Similarly, the genetic control for kernel weight, which determines the differences between maize and its probable progenitor *Z. mays* ssp. *parviglumis*, was studied using QTL mapping (Doebley et al. 1994), which revealed that a QTL on chromosome 3 of maize had large effect on kernel weight and has contributed to early evolution of maize, hence implying that there may have been evolutionary periods during which the fixation of these genes brought relatively rapid change in a reasonably short period of time. Later, Doebley et al. (1995) investigated two QTL controlling differences in plant and inflorescence architecture between maize and its progenitor, one mutant locus *teosinte branchedl* (*tbl*) located on chromosome arm lL reported to influence inflorescence sex and the number and length of internodes in the lateral branches and inflorescences and second QTL, located on chromosome arm X. While the *tb1* locus has strong phenotypic effects in teosinte background, the other QTL had enhanced effect in maize-teosinte F_2 population. The potential of these QTLs to substantially transform inflorescence architecture of both the plants suggests the role of *tb1* locus on morphological diversification of teosinte taxa as well on the domestication of maize. Recently, Studer and Doebley (2012) investigated the occurrence of a natural allelic series for complex traits at the *teosinte branched1* (*tb1*) gene in natural populations of teosinte (*Zea mays* ssp. *parviglumis*, *Z. mays* ssp. *mexicana* and *Z. diploperennis*). The effects of the three allelic classes also correspond to known morphological differences between the teosinte taxa. Likewise in another study, three QTLs on chromosomes 4, 5 and 8 have been identified by using *Z. mays* ssp. *huehuetenangensis* for formation of adventitious roots under flooding conditions (Mano et al. 2005b); three QTLs for controlling roots aerenchyma formation using *Z. nicaraguensis* on chromosomes 1, 5 and 8 (Mano et al. 2006); five QTLs controlling root aerenchyma formation in maize × *Z. luxurians* mapping population (Mano et al. 2008); and five QTLs for root angle in maize × *Z. luxurians* mapping population. The QTL on chromosome 7 influencing second and third root angle was considered most significant for flooding tolerance (Omori and Mano 2007).

2.5.2 Biotechnology Assisted Hybridization

Introgression of variability or novel traits into any crop species requires development of complex hybrids involving distant species (Harlan 1976). Since the complex

hybrids are not stable meiotically, they are difficult to produce through conventional hybridization, and hence the need of specific techniques is required to introgress alien chromatin into the genetic background of crop species. Biotechnology, which includes cell and molecular biology techniques, was developed in the early 1980s. For plant breeders, biotechnology is a powerful tool to assist in introgressive hybridization programmes. Biotechnological tools have helped in overcoming crossing barriers, and using biotechnological tools genes from unrelated sources and even beyond the taxonomic boundaries can be introgressed.

2.5.2.1 Embryo Rescue

The low frequency of hybrid recovery in wide hybridization programmes due to different levels of crossability barriers among maize and its wild relatives is a major hindrance in the corn wide hybridization programmes. The efficiency of hybridization work can be enhanced utilizing the biotechnological tools such as embryo rescue followed by organogenesis. Although, the teosintes are easily crossable with maize under natural conditions, the response of scutella cultures to enhance the efficiency of maize-teosinte hybridization for introgression of desirable traits into genetic background of maize was investigated by Dhaliwal and Lorz (1979). They reported that organogenesis in immature embryos of maize × teosinte (*Z. mexicana*) hybrids resulted in numerous plantlets. The utilization of this biotechnological tool has more significance in the intergeneric hybridizations due to more severe crossability barriers than interspecific crosses leading to low frequency of hybrid recovery. Embryo rescue was used to produce intergeneric maize-*Tripsacum* hybrids by Farquharson (1957). Garcia and Molina (1999) investigated the use of embryo rescue in maize × *Tripsacum* hybrids and in maize × *Zea mays* ssp. *parviglumis* crosses (Garcia and Molina 2001) to enhance the efficiency of wide hybrid production. The response of wide crosses involving a number of maize inbreds as female parents and the morphological characteristics of the hybrids regenerated was evaluated. Nevertheless, the number of hybrids obtained could be increased through the induction of long-term embryogenic callus cultures from the rescued embryos (Furini and Jewell 1995). The success of embryo rescue in intergeneric crosses involving maize and *Tripsacum* was also revealed by Li et al. (1998) and in trihybrid of *Zea mays* × *Zea perennis* × *Zea diploperennis* by Rapela (1984).

2.5.2.2 Somatic Hybridization

Sexual hybridization is the easiest and most successful method of gene transfer; however, it has certain limitations, viz. the plants across taxonomic boundaries cannot be mated and there is no chance of recombination of cytoplasmic genomes. Somatic hybridization through the fusion of somatic protoplasts of two different plant species or varieties allows full genetic recombination involving both nuclear and cytoplasmic genomes. Somatic hybrids were utilized in wide crosses that were

not feasible through sexual hybridization even by utilizing biotechnological tools. The maize protoplasts are isolated from cell suspensions, and the tissue for extraction of protoplasts is selected specifically so as to be able to differentiate later on between somatic hybrids and unfused protoplasts. Polyethylene glycol (PEG) treatment at high pH and high concentration of calcium ions are reported to work well for fusion of maize protoplasts with other protoplasts. The somatic hybrids are selected on the basis of physiological complementation and are singled out from the cell colonies and subcultured followed by their complete regeneration into haploid plants. Somatic hybrids have been reported between maize and *Triticum* sect, *trititrigia* MacKey (*trititrigia*, $2n=35$), a perennial hybrid of *T. durum* Desf. and *Elytrigia intermedium* (host) Nevski by (Wang et al. 1993). The regenerated hybrid plants were identified using restriction patterns of nuclear, ribosomal, mitochondrial and chloroplast genes which revealed that all the hybrids carried only the organellar DNAs of *trititrigia*, which excluded the possibilities of a chimeric callus or any DNA contamination. The potential of gene transfer between *Zea* and *Triticum* species was thus conclusively established. Later, intergeneric somatic hybridization was performed between albino maize protoplasts and mesophyll protoplasts of wheat by PEG treatment. The hybrid plants though sterile despite having male and female flowers were reported to exhibit maize phenotype by Szarka et al. (2002). The cytological analysis of cells from callus tissues and root tips revealed 56 chromosomes, but intact wheat chromosomes were not observed. Genomic in situ hybridization using total wheat DNA as a probe revealed the presence of wheat DNA islands in the maize chromosomal background. Similarly in another study, the flow cytometry analysis of wheat-maize somatic hybrids showed intermediate DNA concentration in hybrid nucleus although; other intermediate morphological traits of plants with hybrid origin were not reported (Göntér et al. 2002). Xu et al. (2003) in a similar experiment tried to combine the genome of wheat and maize in order to generate some valuable breeding material, understand the chromosome elimination mechanism and detect certain interactions between maize and wheat nuclear and cytoplasmic genomes. Unlike earlier workers, they reported that the somatic hybrids were more like wheat instead of maize. Though somatic hybridization seems like a potential alternative for sexual hybridization and success has been achieved in development of hybrid plants, the technique is cumbersome and encompasses limitations at various levels for alien gene transfer owing to difficulty in fusion of protoplasts, fusion of nuclei, elimination of chromosomes and nuclear or cytoplasmic incompatibility.

2.5.3 Direct Gene Transfer

Recombinant DNA techniques have provided plant breeders the opportunities to transfer genes of interest from plants, animals and microbes. Due to the worldwide predominance of cereal grains in the human diet, cereal crops were the prime targets for improvement by genetic transformation. Initially cereal transformation was problematic, due to recalcitrant in vitro cultures, low response to *Agrobacterium-mediated*

transformation and low level activity of some of the promoters in monocot cells and tissues. Maize was among the first cereals in which transgenics were obtained via fusion of recombinant DNA directly in the protoplast cultures derived from immature embryos. The DNA was introduced into the protoplasts by electroporation; a chimeric gene-encoding neomycin phosphotransferase (*neo*), a selectable marker, was introgressed into maize immature embryos or callus (D'Halluin et al. 1992). Also, Silicon carbide whiskers-mediated techniques have also been successfully used to transform maize cell suspension cultures (Frame et al. 1994). Nonetheless, the development of super-binary vector systems contributed to the breakthrough of *Agrobacterium*-mediated transformation of maize (Ishida et al. 1996), for which transformation frequencies of 5–30 % were reported.

2.5.3.1 Herbicide Resistance

Weed flora competes with the crop plants for available nutrients and light energy and thus reduces crop yields by an average of 10–15 %. Through direct gene transfer, it has been possible to provide crop plants with resistance to a certain herbicide, which allows selective elimination of weeds. In general, production of herbicide-resistant crops has involved insertion of only one or two genes that encode inactivation of the herbicide by either overproduction of a herbicide-sensitive biochemical target, structural alteration in biochemical target resulting in altered binding to the herbicide or detoxification or degradation of the herbicide, before it reaches its target site in the plant cell (Stalker et al. 1988). Gordon-Kamm et al. (1990) reported transformation of cells from embryogenic maize suspension cultures with the bacterial gene *bar* encoding for the enzyme phosphinothricin acetyltransferase (PAT) that inactivates the herbicidal compound phosphinothricin (PPT) by acetylation. Fertile transformed maize plants (R) were regenerated, to evaluate the stability of the gene which reduced in the R_1 generation. Likewise now resistance to maize has been incorporated for non-selective herbicides such as glufosinate, glyphosate, imidazolinone, dimethenamide-P, S-metolachlor, flufenacet and terbuthylazine (Zhu et al. 2000; Devos et al. 2008).

2.5.3.2 Insect Resistance

Development of crops that is resistant to insects is expected to increase crop yields since pest infestation can cause reduction in the yield to the tune of up to 10–40 %. This can also save a huge amount of agrochemicals used for crop protection thereby saving considerable amount of money and also the environment. The development of insect-resistant crops was initiated by the discovery that a Gram-positive soil bacterium, *Bacillus thuringiensis* (*Bt*), produces insecticidal crystal proteins (δ-endotoxins) during sporulation. The initial attempts to confer insect resistance by insertion of the gene (*cry*) coding for δ-endotoxin did not provide expected levels of insect resistance due to low *cry* expression, partly due to the high A–T content of the

bacterial gene, which was subsequently modified to fit the higher level G–C content of plant genes, and especially monocot genes. Expression of the altered versions of *cry* genes, *cryIA(b)* and *cryIA(c)*, resulted in a 100-fold higher level of δ-endotoxin production in corn. Transgenic corn expressing the *Bt*-gene *Cry1Ab* was developed in the USA for protection against the devastating European corn borer, *Ostrinia nubilalis,* and was approved for commercial cultivation in the 1990s (Koziel et al. 1993; Armstrong et al. 1995). In elite tropical maize lines, CML67, CML72 and CML216, direct transformation of δ-endotoxin (*cryIAc*) expressing for varying levels of resistance to Southwestern corn borer was carried out by Bohorova et al. (1999). Transgenic corn plant (MON 810), expressing the *Bt* protein, *Cry1Ab*, was reported to provide effective protection against maize stem borer, *Chilo partellus,* even under high level of larval infestation in the greenhouse (Singh et al. 2005). Various Cry proteins entailing resistance genes against insect-pests, namely, *cryIA(b)*, *cryIA(c)*, *cryIF*, *cry3B(b)*, *Cry34Ab1*, *Cry35Ab1*, modified *Cry3A*, *Cry1A.105*, *Cry2Ab2* and *Vip3Aa20,* have been widely used in maize transformation. The *cry* genes exhibit specificity for different insect species, and each protein is active in only one or a few insect species, specificity to a large extent determined by the toxin-receptor interaction, although solubility of the crystal and protease activation also play a role. The members of the *Cry* gene family are grouped in subfamilies according to their specificity for members of the insect families Lepidoptera (caterpillars), Diptera (flies and mosquitoes) and Coleoptera (beetles). Some *Bt* strains have also been reported to be active against other insect families and also mites, nematodes, flatworms and protozoa, but few details as to their practical use are available. It is also significant that several important insect-pests appear to be insensitive to known Cry proteins (e.g. the corn rootworm, aphids and white flies). To overcome this limitation another source of resistance against insect-pests was used, gene *gna* encoding for snowdrop lectin under control of phloem-specific promoter taken from *Galanthus nivalis* L. This toxic agglutinin is toxic to insects such as corn leaf aphid (*Rhopalosiphum maidis* Fitch) under greenhouse conditions (Wang et al. 2005). The toxicity of the endotoxins was significantly enhanced by use of fusion proteins like BtRB, combining the endotoxin cry1Ac with the galactose-binding domain of the nontoxic ricin B-chain (RB) which provides the toxin with additional binding domains, thus increasing the potential number of interactions at the molecular level in target insects. Apart from increased toxicity from the *Bt* gene, the resistance was also transferred against a wider range of insects, including important pests that are not normally susceptible to *Bt* toxins (Mehlo et al. 2005).

2.5.3.3 Abiotic Stress Tolerance

Drought is a major abiotic stress that limits crop productivity and engineering plants with enhanced tolerance of abiotic stresses such as drought is a major objective of plant biotechnology that is expected to be commercialized in the near future. Tolerance to abiotic stress may be achieved through the modification of endogenous plant pathways, often by manipulating important regulatory proteins such as

transcription factors. Altering the level of expression of key transcription factors involved in abiotic stress pathways has been shown to enhance tolerance to drought stresses in maize (Nelson et al. 2007). Van Breusegem et al. (1999a, b) reported introgression of transgenes expressing for manganese superoxide dismutase that entails foliar tolerance to chilling and oxidative stress and for iron superoxide dismutase entailing enhanced tolerance towards methyl viologen and increased growth rates, respectively.

2.5.3.4 Biofortification

Micronutrient deficiency in developing nations, especially where populations subsist on a monotonous diet of cereal grains that lack essential vitamins and minerals, is a major challenge to health organizations and governments throughout the world, with an estimated 40–50 % of the world's population suffering at one time from diseases caused by a lack of such essential minerals and vitamins. Pyramiding of genes controlling the nutritional quality is difficult via sexual hybridization because large number of loci control the pathway of nutrient accumulation in the plants. Hence, biofortification of staple food grains can be an effective way to provide essential nutrients to consumers whose diets rely heavily on these grains. Biofortification of maize has been carried out for vitamins, iron and zinc. Vitamin deficiency affects up to 50 % of the world's population. Transgenic plants offer an effective way to increase the vitamin content of staple crops, but thus far it has only been possible to enhance individual vitamins. Transgenic corn plants have been developed with the levels of β-carotene, ascorbate and folate vitamins increased specifically in the endosperm to the tune of 169-, six- and twofold, respectively, through the simultaneous modification of three separate metabolic pathways (Naqvi et al. 2009). Similarly, Drakakaki and co-workers (2005) generated transgenic maize expressing both an *Aspergillus* phytase and soybean ferritin in the kernel increasing the concentration of Fe up to 50 % along with 95 % of phytate degradation directly correlated with Fe bioavailability and uptake. Biofortification of maize was also carried out for amino acids lysine and tryptophan, reduction in bitter-tasting sinapinic acid and raising the content of vitamin E.

2.6 Impact of Alien Gene Transfer in Maize

Maize being an allogamous crop promotes heterozygosity and hence possesses genetic plasticity in the populations. Therefore, it has an advantage over any other crop in terms of yield potential, adaptability and natural genetic variability. The evolutionary developments and natural hybridization between maize and its relatives in isolated niches have contributed a lot in the available genetic diversity in its primary gene pool. Nonetheless, maize exhibits vast potential for introgression of alien genes due to availability of diverse sources of genes belonging to seven

species and subspecies of genus *Zea* and 16 species of genus *Tripsacum*. The transfer of desirable genes has been successfully achieved via utilizing both conventional and modern biotechnology (Repellin et al. 2001). Introgressive hybridization programmes involving alien genes are extensively time consuming and result in development of breeding lines that are further used in breeding programmes instead of having direct use as commercial cultivar. Use of alien genes has been reported for improvement of agronomic and yield traits; resistance to disease, insect-pests and parasitic weeds; and tolerance to abiotic stresses through conventional hybridization methods. Likewise, biotechnological tools were also employed for the purpose among which the most successful method with practical implication was genetic engineering of maize through recombinant DNA technology. Genetically modified maize covers 42 million ha area contributing to a total of 26 % of cultivated area under maize throughout world.

Genetic engineering has allowed incorporation of traits hitherto not available in the crop germplasm, viz. herbicide resistance. A number of maize cultivars are commercially available with resistance against otherwise non-selective herbicides. Nevertheless, the herbicide-tolerant corn in field has sparked a new debate over the risk of transfer of these herbicide-resistant genes to wild species and hence leading to development of superweeds (Devos et al. 2008). Similarly the insect-resistant genetically modified crop is being cultivated throughout the world and is successful in controlling insect-pests, saving tremendous cost on production and use of harmful chemicals and also having a positive environmental impact. However, there are concerns on the other side of using the transgenic maize varieties as it is opined that such transgenes are affecting the nontarget species also is considered as the negative aspect of this technique. The most useful facet of genetic engineering lies in the biofortification of maize for increasing the amount and availability of minerals and vitamins for which this crop is otherwise deficient. Biofortified maize has provided nutritional security to the people who are solely dependent on corn as staple food. Likewise, genetic engineering of corn has also been focussed towards increasing the content of bio-products like bioethanol or amylase and production of plastics, which are of industrial importance. The use of maize as industrial raw material has further enhanced its value as a cultivated crop.

2.7 Conclusion

Since the commencement of systematic maize improvement endeavours, the breeding objectives were to attain high productivity which were well exploited using available variability within the crop germplasm. However, uniformity and narrow genetic base among the commercial cultivars and their parents served as a bottleneck in genetic upgradation of maize for which distant hybridization programmes were taken up for introgression of desirable alien genes. The prevalence of various fertilization barriers has reduced the possibility of alien introgressions into the cultivated background of maize through sexual hybridization. To overcome the sexual barriers

encountered during wide hybridization endeavours, biotechnological tools have assisted the maize breeders to a great extent. The utilization of recombinant DNA technology for genetic engineering of corn has not only helped in increasing yield but has also provided nutritional security and industrial advantage. Conclusively, the transfer of alien genes either through conventional or biotechnological methods has contributed significantly in enhancing the value of this crop.

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Chapter 3
Oat

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Abstract Oat (*Avena sativa* L.) productivity is affected by crown rust (*Puccinia coronata* f. sp. *avenae*) and stem rust (*Puccinia graminis* f. sp. *avenae*) worldwide. Control of these diseases has been through the use of host resistance genes, but frequent changes in pathogen virulence provide a continuing threat to oat production. Wild oat species have been a major source of diversity for the improvement of cultivated oat. Many rust resistance genes, as well as genes providing resistance to other major oat diseases, have been found in wild oat species, landraces as well as in cultivated species and have been utilised in plant breeding. However, the transfer of resistance from wild diploid and tetraploid species to cultivated hexaploid oat is difficult because their chromosomes do not pair readily. Nevertheless, many improved oat cultivars possess alien-derived rust resistance genes and occupy considerable acreage in the major oat-producing regions of the world. This chapter reviews the major developments and their impacts on oat breeding, specifically through alien gene transfer from wild and related species.

Keywords Alien gene transfer • *Avena* • Disease resistance • Rust • Oat • Wild species

3.1 Introduction

Oat (*Avena sativa* L.) is an ancient cereal crop which has been cultivated at least since the time of Theophrastus (371–286 BC) and grown worldwide (Martens 1985). The genus *Avena* consists of diploid ($2n=2x=14$), tetraploid ($2n=4x=28$) and

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hexaploid ($2n=6x=42$) species, with a basic chromosome number of seven ($x=7$) (Thomas 1992). The hexaploid *Avena* genome is comprised of three subgenomes (A, C and D), which represents modifications of a common homologous chromosome series (Rajhathy and Thomas 1974). Diploid and tetraploid species are classified into A, B, C and D genome groups, with further subdivisions based on karyotypes and chromosome pairing in interspecific hybrids. The cultivated white hexaploid oat (*A. sativa* L.) spread from the Near East to Europe in the late Bronze Age, while the cultivated red oat (*A. byzantina* C. Koch) was the main form in North Africa and in Spain. Both species were introduced into North America in the sixteenth century (Coffman 1977).

One of the major constraints to oat production worldwide is plant disease (Harder and Haber 1992). Among several diseases that attack oat, crown rust and stem rust are the most damaging, affecting both yield and quality of oat grain and forage. Up to 35 % yield loss due to severe stem rust infection has been found in Canada (Martens 1978), while up to 50 % loss due to crown rust infection has been estimated in the United States (Simons 1985). Both rust diseases have primarily been controlled through the use of host resistance genes (McCallum et al. 2007). Many accessions of wild hexaploid and diploid oat have been found to be sources of desirable rust resistance genes for incorporation into cultivated oat. The identification of wild accessions with effective resistant genes is an important step in the process of developing locally adapted cultivars with improved crown rust and stem rust resistance. The genetic characterisation of rust resistance genes in *Avena* species has been carried out by researchers since the 1920s.

Parker (1920) was one of the earliest workers to investigate crown rust resistance in oat. He recognised the importance of determining the inheritance of rust resistance to plant breeding, and that linkage of crown rust resistance with undesirable traits could be a hindrance to crop improvement. Rust resistance types have been categorised broadly into specific (major, vertical, seedling, etc.) or general (minor, horizontal, partial or adult plant resistance (APR)). Specific resistance, usually governed by single genes, is expressed at all growth stages, and, while relatively easy to identify and incorporate into elite germplasm, has been frequently overcome by rust races with matching virulence. Partial resistance, usually conferred polygenically, does not prevent infection completely but reduces pustule size and numbers of spores produced and extends the latency period of pustule development (Portyanko et al. 2005). Partial resistance may be more durable than specific resistance because there is less selection pressure on the pathogen and therefore slows the evolution of virulence (Simons 1972). However, partial resistance is more difficult to use in plant breeding since several genes need to be incorporated, and is evaluated at the adult plant stage. Thus, specific resistance genes are desirable in plant breeding. While most of the hexaploid oat germplasm has been exploited in the search for new rust resistance genes, this chapter reviews the utilisation of alien genes to enhance rust resistance in oat.

3.2 Crown Rust

Crown rust, caused by the fungus *Puccinia coronata* f. sp. *avenae*, is a widespread and damaging disease of oat. Oat crown rust infection is favoured by periods of high leaf moisture (rainfall or dew) and moderate temperatures (21–25 °C) during the growing season (Carson 2011). Crown rust occurs commonly on wild *A. fatua* in the United States and Canada (Leonard 2003; Chong et al. 2011) and on *A. sterilis* and other wild oat species in the Mediterranean regions of southern Europe, Northern Africa and the Middle East (Zillinsky and Murphy 1967). Losses in both yield and grain quality commonly result from epidemics of crown rust (Simons 1985), with individual oat fields suffering total crop failure. Crown rust generally attacks the leaves and interferes with transport of photosynthesised sugars from leaves to the developing grain, which causes shrivelled grain with reduced quality. Badly rusted plants develop stunted root systems and have poor drought tolerance. Control of crown rust disease can be attained using fungicides, but disease resistance is the most effective, cost-efficient and environment friendly control method.

Breeding for resistance to crown rust began in 1919 (Simons 1985), and to date over 100 alleles have been described (Table 3.1). Crown rust resistance genes have

Table 3.1 Oat crown rust resistance genes

Gene	Original source	*Avena* species	Reference
Pc1	Red Rustproof	*A. byzantina*	Dietz and Murphy (1930)
Pc2	Victoria	*A. byzantina*	Murphy et al. (1937)
Pc2b	Anthony/Bond/Boone		Finkner (1954)
Pc3	Bond	*A. byzantina*	Hayes et al. (1939)
Pc3c	Ukraine	*A. sativa*	Weetman (1942)
Pc4	Bond	*A. byzantina*	Hayes et al. (1939)
Pc4c	Ukraine	*A. sativa*	Weetman (1942)
Pc5	Landhafer	*A. byzantina*	Litzenberger (1949)
Pc6	Santa Fe	*A. byzantina*	Litzenberger (1949)
Pc6c	Ukraine	*A. sativa*	Finkner (1954)
Pc6d	Trispernia	*A. sativa*	Finkner (1954)
Pc7	Santa Fe	*A. byzantina*	Osler and Hayes (1953)
Pc8	Santa Fe	*A. byzantina*	Osler and Hayes (1953)
Pc 9	Ukraine	*A. sativa*	Finkner (1954)
Pc9c	Santa Fe	*A. byzantina*	Simons and Murphy (1954)
Pc10	Klein 69B	*A. byzantina*	Finkner (1954)
Pc11	Victoria	*A. byzantina*	Welsh et al. (1954)
Pc12	Victoria	*A. byzantina*	Welsh et al. (1954)
Pc13	Clinton	*A. sativa*	Finkner et al.(1955)
Pc14	Ascencao	*A. byzantina*	Simons (1956)
Pc15	Saia	*A. strigosa*	Murphy et al. (1958)

(continued)

Table 3.1 (continued)

Gene	Original source	*Avena* species	Reference
Pc16	Saia	*A. strigosa*	Murphy et al. (1958)
Pc17	Saia	*A. strigosa*	Murphy et al. (1958)
Pc18	Glabrota	*A. glabrota*	Simons et al. (1959)
Pc19	CI 3815	*A. strigosa*	Simons et al. (1959)
Pc20	CI 7233	*A. abyssinica*	Simons et al. (1959)
Pc21	Santa Fe	*A. byzantina*	Chang (1959)
Pc22	Ceirch du Bach	*A. sativa*	McKenzie (1961)
Pc23	C.D 3820	*A. strigosa*	Dyck and Zillinsky (1962)
Pc24	Garry	*A. sativa*	Upadhyaya and Baker (1960)
Pc25	Garry	*A. sativa*	Upadhyaya and Baker (1960)
Pc26	Garry	*A. sativa*	Upadhyaya and Baker (1960)
Pc27	Garry	*A. sativa*	Upadhyaya and Baker (1960)
Pc28	Garry	*A. sativa*	Upadhyaya and Baker (1960)
Pc29	Glabrota	*A. glabrota*	Marshall and Myers (1961)
Pc30	CI 3815	*A. strigosa*	Marshall and Myers (1961)
Pc31	CI 4746	*A. strigosa*	Marshall and Myers (1961)
Pc32	CeirchLlwyd	*A. strigosa*	Marshall and Myers (1961)
Pc33	CeirchLlwyd	*A. strigosa*	Marshall and Myers (1961)
Pc34	D-60	*A. sterilis*	McKenzie and Fleischmann (1964)
Pc35	D-137	*A. sterilis*	McKenzie and Fleischmann (1964)
Pc36	CI 8081	*A. sterilis*	Simons (1965)
Pc37	CD 7994	*A. strigosa*	Dyck (1966)
Pc38	CW 491-4	*A. sterilis*	Fleischmann and McKenzie (1968)
Pc39	F-366	*A. sterilis*	Fleischmann and McKenzie (1968)
Pc40	F-83	*A. sterilis*	Fleischmann and McKenzie (1968)
Pc41	F-83	*A. sterilis*	Fleischmann and McKenzie (1968)
Pc42	F-83	*A. sterilis*	Fleischmann and McKenzie (1968)
Pc43	F-83	*A. sterilis*	Fleischmann and McKenzie (1968)
Pc44	Kyto	*A. sativa*	Martens et al. (1968)
Pc45	F-169	*A. sterilis*	Fleischmann et al. (1971a)
Pc46	F-290	*A. sterilis*	Fleischmann et al. (1971a)
Pc47	CI 8081A	*A. sterilis*	Fleischmann et al. (1971b)
Pc48	F-158	*A. sterilis*	Fleischmann et al. (1971b)
Pc49	F-158	*A. sterilis*	Fleischmann et al. (1971b)
Pc50	CW 486	*A. sterilis*	Fleischmann et al. (1971b)
Pc51	Wahl No.8	*A. sterilis*	Simons et al. (1978)
Pc52	Wahl No.2	*A. sterilis*	Simons et al. (1978)
Pc53	6-112-1-15	*A. sterilis*	Simons et al. (1978)
Pc54	CAV 1832	*A. sterilis*	Simons et al. (1978)
Pc55	CAV 4963	*A. sterilis*	Kiehn et al. (1976)
Pc56	CAV 1964	*A. sterilis*	Kiehn et al. (1976)
Pc57	CI 8295	*A. sterilis*	Simons et al. (1978)
Pc58	PI 295919	*A. sterilis*	Simons et al. (1978)
Pc59	PI 296244	*A. sterilis*	Simons et al. (1978)
Pc60	PI 287211	*A. sterilis*	Simons et al. (1978)

(continued)

Table 3.1 (continued)

Gene	Original source	*Avena* species	Reference
Pc61	PI 287211	*A. sterilis*	Simons et al. (1978)
Pc62	CAV 4274	*A. sterilis*	Harder et al. (1980)
Pc63	CAV 4540	*A. sterilis*	Harder et al. (1980)
Pc64	CAV 4248	*A. sterilis*	Wong et al. (1983)
Pc65	CAV 4248	*A. sterilis*	Wong et al. (1983)
Pc66	CAV 4248	*A. sterilis*	Wong et al. (1983)
Pc67	CAV 4656	*A. sterilis*	Wong et al. (1983)
Pc68	CAV 4904	*A. sterilis*	Wong et al. (1983)
Pc69	CAV 1387	*A. sterilis*	Harder et al. (1984)
Pc70	PI 318282	*A. sterilis*	CDL (2010)
Pc71	IA B437	*A. sterilis*	CDL (2010)
Pc72	PI 298129	*A. sterilis*	CDL (2010)
Pc73	PI 309560	*A. sterilis*	CDL (2010)
Pc74	PI 309560	*A. sterilis*	CDL (2010)
Pc75	IB 2402	*A. sterilis*	Fox et al. (1997)
Pc76	IB 2465	*A. sterilis*	Fox et al. (1997)
Pc77	IB 2433	*A. sterilis*	Fox (1989)
Pc78	IB 1454	*A. trichophylla*	Fox (1989)
Pc79	IB 1454	*A. trichophylla*	Fox et al. (1997)
Pc80	IB 3432	*A. sterilis*	Fox (1989)
Pc81	CI 3815	*A. strigosa*	Yu and Wise (2000)
Pc82	CI 3815	*A. strigosa*	Yu and Wise (2000)
Pc83	CI 3815	*A. strigosa*	Yu and Wise (2000)
Pc84	CI 3815	*A. strigosa*	Yu and Wise (2000)
Pc85	CI 3815	*A. strigosa*	Yu and Wise (2000)
Pc86	CI 3815	*A. strigosa*	Yu and Wise (2000)
Pc87	CI 3815	*A. strigosa*	Yu and Wise (2000)
Pc88	CI 3815	*A. strigosa*	Yu and Wise (2000)
Pc89	CI 3815	*A. strigosa*	Yu and Wise (2000)
Pc90	CI 3815	*A. strigosa*	Yu and Wise (2000)
Pc91	CW 57	*A. longiglumis*	Rooney et al. (1994)
Pc92	Obee/Midsouth	*A. strigosa*	Rooney et al. (1994)
Pc93	CI 8330		CDL (2010)
Pc94	RL 1697	*A. strigosa*	Aung et al. (1996)
Pc95	Wisc X 1588-2	*A. sativa*	Harder et al. (1995)
Pc96	RL 1730	*A. sativa*	Chong and Brown (1996)
Temp_*Pc97*	CAV 1180	*A. sterilis*	Chong et al. (2011)
Temp_*Pc98*	CAV 1979	*A. sterilis*	Chong et al. (2011)

been identified from four main sources: *A. byzantina*, *A. sativa*, *A. sterilis* and lower ploidy material (diploid oat, mainly *A. strigosa*). The best sources of genes for resistance to *P. coronata* can be found among wild relatives of oat in the regions of its origin (Manisterski and Wahl 1995). Of the wild relatives, *A. sterilis* is the most important source of resistance genes (Segal et al. 1980; Simons 1985) and can be

readily hybridised with cultivated oat. Although nearly 30 genes have been described in lower ploidy wild oat, relatively few have been used in oat breeding. Crown rust resistance genes (*Pc* genes) developed from the various sources are listed below.

3.2.1 A. byzantina

The first early successes in finding crown rust resistance genes were from *A. byzantina* introductions. *Pc1*, a dominant gene, was isolated from the cultivar Red Rustproof (Davies and Jones 1927), designated "S" by Dietz and Murphy (1930). Victoria, an introduction from Uruguay, was found to contain three genes: *Pc2* (Murphy et al. 1937), *Pc11* and *Pc12* (Welsh et al. 1954). Bond, from Red Algerian/Gold Rain out of Australia (Welsh et al. 1953), contained two dominant complementary genes for crown rust resistance: *Pc3* and *Pc4* (Hayes et al. 1939). *Pc5* was a gene found in Landhafer (Litzenberger 1949) and was an introduction from Germany, but it probably originated in South America (Coffman 1961). Another South American introduction was Santa Fe, which was the source of three resistance genes: *Pc6* (Litzenberger 1949), *Pc7* and *Pc8* (Osler and Hayes 1953). Klein was an *A. byzantina* introduction from Argentina which came to North America from Australia (Welsh et al. 1953), and Klein 69b was found to contain *Pc10* (Finkner 1954). Simons (1956) identified two genes in the variety Ascencao: one was determined to be *Pc2* while the second, *Pc14*, was shown to be epistatic to *Pc2*.

3.2.2 A. sativa

It would appear that *A. sativa* contains relatively few naturally occurring resistance genes useful against *P. coronata*. Ukraine (Hutica), an introduction from Russia, contained two dominant complementary genes, *Pc3c* and *Pc4c* (Weetman 1942). Finkner (1954) showed that two closely linked alleles, *Pc6c* and *Pc9*, conferred resistance in Ukraine. But a later work by Sanderson (1960) showed that Ukraine resistance was due to a single dominant gene, *Pc9*. Trispernia, an introduction from Romania (Welsh et al. 1953), contained three genes for resistance, one of which was *Pc6d* (Finkner 1954). *Pc13* was found to be present in the widely grown US oat cultivar Clinton (Finkner et al. 1955). *Pc22* was isolated from the Welsh variety Ceirch du Bach (McKenzie 1961). The cultivar Garry in Canada was found by Upadhyaya and Baker (1960) to possess three seedling genes (*Pc24*, *Pc25*, *Pc26*) and two APR genes (*Pc27*, *Pc28*). Kyto oat was found to contain the dominant gene *Pc44* (Martens et al. 1968). More recently, *Pc96* was identified in *A. sativa* (Chong and Brown 1996) and is currently effective against predominant races of *P. coronata* in North America (Chong et al. 2011), thus is useful for combining with other effective *Pc* genes.

3.2.3 *A. sterilis*

A. sterilis is a wild hexaploid oat species which is native in the Mediterranean regions (Wong et al. 1983). Accessions were collected in Israel and other Mediterranean countries during the 1960s and the early 1970s (Simons et al. 1978). The transfer of resistance from wild hexaploid *A. sterilis* to cultivated oat, *A. sativa*, was reported to be more successful than from other related wild species (Martens and Dyck 1989). *A. sterilis* has been the richest source of crown rust resistance genes of all species. To date there have been 43 genes described, many of which are linked or allelic (Table 3.1). Most *Pc* genes derived from *A. sterilis* have been defeated in North America, due to single gene deployment and rapid virulence changes in the *P. coronata* population. For example, *Pc68* was derived from *A. sterilis* and was one of the most effective major resistance genes deployed globally against crown rust, but virulence to this gene is now common in North America. Most of the *A. sterilis* genes for crown rust resistance are dominant or incompletely dominant, but there are exceptions as *Pc54* was shown to be an incompletely recessive gene (Martens et al. 1980).

At least 16 genes from *A. sterilis* have been deployed at various times in North America (Leonard 2003). *Pc38*, *Pc39* and *Pc68*, and to a lesser extent *Pc48*, have been used in developing resistant oat cultivars in the eastern prairie region of Canada. *Pc39* was the first gene derived from *A. sterilis* to be deployed in Canada, with the release of "Fidler" in 1980. This was followed by the release of a series of cultivars (such as "Dumont", "Robert" and "Riel") with both *Pc38* and *Pc39* from 1982 to 1993. *Pc38* and *Pc39* became ineffective in the eastern prairie region in the mid-1990s, due to a major shift in virulence to these two genes in the prairie rust population (Chong and Seaman 1997). Subsequently, a series of cultivars with the *Pc38 + 39 + 68* gene combination were released, starting with "AC Assiniboia" in 1995, followed by six other cultivars with the same gene combination. Since *Pc38* and *Pc39* were no longer effective in the 1990s, these cultivars basically were protected only by *Pc68*. In 1998, "Triple Crown" was released and its resistance was based on gene *Pc48*. In 2001, late-planted fields of this cultivar were severely damaged by crown rust (Chong and Zegeye 2004). By 2005 cultivars with the *Pc38*, *Pc39*, *Pc68* gene combination ("AC Assiniboia" and "Ronald") were severely damaged by crown rust (Chong et al. 2008), indicating that *Pc68* was no longer effective in Canada. Since then, two putative new genes from *A. sterilis* (*Temp_Pc97* and *Temp_Pc98*) have been described and are resistant to most current *P. coronata* races in Canada (Chong et al. 2011).

3.2.4 *Diploid and Tetraploid Oat*

Several wild diploid species of *Avena*, particularly *A. strigosa*, possess a high degree of resistance to crown rust. *A. strigosa* is primarily found in Western Europe and in

countries of the former USSR. This species was widely cultivated for grain-fodder (Holden 1979) and is currently grown to a limited extent in Germany, the United Kingdom (Wales) and Australia. It is also used as a fodder oat in Brazil (Leonard and Martinelli 2005). Transfer of genes for crown rust resistance from *A. strigosa* to hexaploid oat was initially accomplished by producing an autotetraploid of *A. strigosa* followed by crossing to *A. sativa* (Zillinsky and Derick 1960). However, the resistant hexaploid lines isolated in the later generations exhibited a high degree of sterility and cytological instability (Dyck and Zillinsky 1963). Wild diploid *A. strigosa* accessions have also been found to be a better source of adult plant resistance when compared to accessions of the tetraploid species, *A. barbata* (Cabral et al. 2011). There have been 22 genes described from *A. strigosa* (Table 3.1). Of these genes, only *Pc23* and *Pc94* have been incorporated into a stable *A. sativa* background (Dyck and Zillinsky 1963; Aung et al. 1996).

The lack of effective crown rust resistance genes in cultivated oat and the ability of the pathogen to produce new races have resulted in the continuous search for new sources of resistance. The useful lifetime of a resistance gene can be relatively short due to changes in virulence in the pathogen population following widespread deployment of an introduced resistance gene. Early oat breeding efforts incorporated resistance genes from cultivated hexaploid oat (Martens and Dyck 1989). When all available sources of resistance were defeated by the late 1950s, new sources of crown rust resistance were identified in *A. sterilis*. More than 40 resistance genes have been described, but few are useful for oat breeding. Moderate levels of virulence already existed in the *P. coronata* f. sp. *avenae* population to many of these genes, even though they had not previously been deployed in North America (Chong and Kolmer 1993). Some of the genes were tightly linked or allelic, and virulence to some of the genes was associated (Chong and Brown 1996; Leonard et al. 2005b). Few crown rust genes have been described from wild diploid and tetraploid species, even though they are a rich source of genetic diversity (Simons et al. 1959). *Pc91* is a highly effective gene derived from a cross between tetraploid *A. magna* Murphy and Terrell and diploid *A. longiglumis* Durr. (Rooney et al. 1994), with *A. magna* being the donor. *Pc91* is currently the most effective crown rust resistance gene available in North America (McCartney et al. 2011). *Pc94* was derived from *A. strigosa* (Aung et al. 1996) and was highly effective during 2002–2006 (Chong et al. 2008). Currently, oat breeders are interested in pyramiding *Pc91* and *Pc94* to slow the breakdown of these resistance genes. Pyramiding these genes in conjunction with APR genes may stabilise crown rust resistance.

3.3 Stem Rust

Stem rust is another major disease that threatens oat production worldwide. The disease is caused by the fungus *Puccinia graminis* Pers. f. sp. *avenae* Eriks. and E. Henn. The most severe epidemic recorded in Canada caused an estimated 35 % yield loss in the eastern prairie region in 1977 (Martens 1978). The most recent

Table 3.2 Oat Stem rust resistance genes

Gene	Original source	*Avena* species	Reference
Pg1	White Russian	*A. sativa*	Garber (1921)
Pg2	Green Russian	*A. sativa*	Dietz (1928)
Pg3	Joanette	*A. sativa*	Waterhouse (1930)
Pg4	HajiraRL1225	*A. sativa*	Welsh and Johnson (1954)
Pg5	RL 1225	*A. sativa*	Welsh and Johnson (1954)
Pg6	CD 3820	*A. strigosa*	Murphy et al. (1958)
Pg6a	CN 21997, CN 57130	*A. strigosa*	Zegeye 2008
Pg6b	CN 21996	*A. strigosa*	Zegeye 2008
Pg6c	CN 21998, CN 22000	*A. strigosa*	Zegeye 2008
Pg6d	CN 22001	*A. strigosa*	Zegeye 2008
Pg6e	CN 55115	*A. strigosa*	Zegeye 2008
Pg7	CD 3820	*A. strigosa*	Murphy et al. (1958)
Pg8	Hajira CI 8111	*A. sativa*	Browning and Frey (1959)
Pg9	Ukraine, Santa Fe	*A. sativa*	McKenzie and Green (1965)
Pg10	Illinois Hulless, CI 2824	*A. sativa*	Pavek and Myers (1965)
Pg11	Burt, CI 3034	*A. sativa*	McKenzie and Martens (1968)
Pg12	Kyto, CI 8250	*A. sativa*	Martens et al. (1968)
Pg13	PI 324798, CW 490-2	*A. sterilis*	McKenzie et al. (1970)
Pg14	Milford	*A. sativa*	Mac Key and Mattsson (1972)
Pg15	CAV 1830	*A. sterilis*	Martens et al. (1980)
Pg16	D203	*A. barbata*	Martens et al. (1979)
Pg17	IB 3056	*A. sterilis*	Harder et al. (1990)
Pg-a	*A. sterilis*/Kyto	*A. sterilis* + *sativa*	Martens et al. (1981)

epidemic of oat stem rust in Canada occurred in the eastern prairie region in 2002, with estimated yield losses of 6.6 % (worth $12.6 million) in Manitoba and 0.5 % (worth $1.0 million) in Saskatchewan (Fetch 2005). The disease can be effectively controlled with host resistance or with fungicides. However, fungicides can be an expensive option for producers besides having environmental impacts. Therefore, the most efficient, economical and environmentally sound method of controlling the disease is through the use of host resistance.

Currently, only 17 oat stem rust resistance genes (*Pg* genes) and the *Pg-a* complex have been described (Martens 1985; Fetch and Jin 2007) and are listed in Table 3.2. Most are from *A. sativa*, but a few are from wild oat (Martens 1985). Oat stem rust resistance genes are rare (Martens and Dyck 1989), and only a few genes (*Pg2*, *Pg13*, *Pg-a*) provide protection against the current races in the North American population of *P. g*. f. sp. *avenae* (Mitchell Fetch and Fetch 2011). Harder (1994) reported that only five genes (*Pg1*, *Pg2*, *Pg4*, *Pg9* and *Pg13*) had been intentionally deployed in Canadian oat cultivars, and most resistant cultivars likely possessed the *Pg2* + *Pg13* combination. This combination was effective against all stem rust races in North America, but new races (NA67, NA76) with virulence to these genes evolved and are common in North America (Fetch and Dunsmore 2004).

Virulence in the population of *P. g*. f. sp. *avenae* appears to be increasing (Mitchell Fetch and Fetch 2011); thus new genes are highly desirable. Only *Pg6*, *Pg10*, *Pg11*, *Pg12*, *Pg16* and the *Pg-a* complex confer resistance to NA67 and NA76 (Fetch and Dunsmore 2004). Genes *Pg10*, *Pg11*, *Pg12* and the *Pg-a* complex are from a hexaploid background. To date, only the *Pg-a* complex has been deployed in oat cultivars in Canada and the United States, but the frequency of virulent races to *Pg-a* is increasing in North America. The *Pg10* gene has been used in Canadian oat breeding programmes, but no cultivars have been developed to date. This gene confers characteristic large necrotic halos around rust pustules and on stems of adult plants (Harder 1999), and while no virulence has been detected in North America, there is virulence to *Pg10* in Ethiopia (Fetch, unpublished data). Gene *Pg11* is an APR gene and is effective to all races of oat stem rust, but is associated with chlorophyll deficiency (Harder et al. 1971) and is undesirable for breeding. Gene *Pg12* is effective only at the seedling stage (Martens 1985).

Since the virulence spectrum in *P. g*. f. sp. *avenae* is increasing and most known genes are either ineffective or undesirable, new genes are needed. A recent study evaluated nearly 10,000 lines from various species of *Avena* for resistance to race NA67. Results indicated that *A. strigosa* is the most promising source of resistance and that additional resistance genes in hexaploid oat are unlikely to be found (Gold Steinberg et al. 2005). Gene *Pg16* has been successfully transferred from the tetraploid *A. barbata* into hexaploid oat by irradiation (Brown 1985); however it is a 44-chromosome addition line and appears to reduce yield by about 10 % (J. Mitchell Fetch, unpublished). Efforts to reduce the chromosome number to 42 and maintain the resistance have not been successful. Gene *Pg6* derives from the diploid species *A. strigosa* and is resistant to most races of oat stem rust in North America except BLD (NA1) and CLD (NA70) (Fetch and Jin 2007). This gene reportedly was transferred into the American oat cultivar "Delredsa" (Rothman 1984), but multipathotype tests indicated *Pg6* was not present (Fetch, unpublished data). Recent efforts have been made by Zegeye (2008) to transfer resistance identified in the Gold Steinberg et al. (2005) study from *A. strigosa* into *A. sativa*. Efforts were partially successful, but resistant lines were chromosome addition lines, and no hexaploid derivatives containing *A. strigosa* resistance to oat stem rust has yet been developed.

3.4 Transfer of Rust Resistance from Wild Oat Accessions to Common Oat

There are many sources of rust resistance from diploid and tetraploid wild oat species. However, while transferring resistance from lower ploidy material to hexaploid wheat has been highly successful (Knott 1987), this has not been realised in oat. There are seven different genomes identified in diploid oat species and at least three in tetraploid species (Rajhathy and Thomas 1974). These genomes have differing affinities for pairing with the *A. sativa* genome, thus developing meiotically

stable progeny from interploidy crosses has been difficult. Interploidy transfer, particularly from diploid to hexaploid genomes, often requires special manipulations such as embryo rescue or development of a synthetic hexaploid, which can be crossed to a hexaploid cultivar (Innes and Kerber 1994). For example, *Pc91* is a highly effective gene in hexaploid oat that was transferred using a synthetic hexaploid derived from a cross between tetraploid *A. magna* Murphy and Terrell and diploid *A. longiglumis* Durr. (Rooney et al. 1994). In oat, diploid to hexaploid resistance gene transfers have also been facilitated by generation of autotetraploid, derived tetraploid and amphiploid lines (Sadanaga and Simons 1960). In contrast, the transfer of rust resistance genes from wild *A. sterilis* is easy as this species pairs readily with *A. sativa*.

The process of resistance gene transfer from wild to cultivated oat is often hindered by sterility barriers (Aung et al. 1977). Thus, generation of fertile progeny may involve the rescue of hybrids or F_1 embryos. The choice of the female and pistillate parent in interploidy crosses is also an important consideration, with the lower ploidy genotype being the preferred pistillate parent (Rajhathy and Thomas 1974). On the contrary, retaining the higher ploidy hexaploid as the pistillate parent yielded more vigorous F_1 seeds from interspecific crosses of hexaploid oat cv. "Wintaroo" and diploid *A. strigosa* genotypes (Cabral et al. 2013). Additionally, instances of suppressor genes/factors of the donor parent interfering with the expression of resistance in interspecific F_1 progeny have been reported. Rines et al. (2007) reported a suppressor factor in diploid line CI6954SP that contributed to susceptible F_1 progeny when crossed with *A. sativa*. The gene*Pc38* suppresses the expression of *Pc94* (Aung et al. 1996), which has been introgressed into *A. sativa* from *A. strigosa*.

Even if generation of fertile progeny from interspecific crosses is achieved, the introgression of resistance into the genome of hexaploid oat cultivars is very difficult to achieve by means of regular backcrossing procedures. This is mainly due to the low frequency of chromosome pairing between wild species with cultivated oat, consequently reducing the chance of recombination and gene transfer. Rajhathy and Thomas (1974) reported that a genotype of *A. longiglumis* (CW57) suppressed the activity of the gene(s) controlling regular bivalent pairing in *A. sativa* and induced pairing between nonhomologous chromosomes, resulting in translocation between two homologous chromosomes as well as between unrelated chromosomes. The CW57 gene(s) induced pairing between line RL1697 (*A. strigosa*) and line Sun II (*A. sativa*), which facilitated the transfer of *Pc94* crown rust into a hexaploid genetic background (Chong et al. 2011).

Though the transfer of resistance genes from wild species into hexaploid oat is difficult, several success stories and methods have been reported. Ladizinsky (1995) described the domestication of two wild tetraploid oat species not by selection of rare mutations but by transfer of genes into cultivated oat through hybridisation. He later developed a synthetic hexaploid oat (2000) by crossing the *A. strigosa* ($2n = 14$) cv. "Saia" with *A. magna* ($2n = 28$). Chromosome doubling of the resulting sterile triploid hybrid produced a synthetic hexaploid, which was intermediate between its parents in panicle shape and lemma colour. Progeny were similar to the tetraploid parent in spikelet structure and to the diploid parent in having a single, albeit

partially shrivelled seed per spikelet and low protein content. Fox (1989) crossed three tetraploid accessions with Rodney O (hexaploid *A. sativa*) and produced highly fertile progeny with resistance to crown rust races CR13 and CR 50, but two diploid accessions crossed with Rodney O did not produce any viable seeds.

Zillinsky and Derick (1960) first reported the transfer of genes from wild diploid *A. strigosa* into hexaploid oat. They created an autotetraploid line by doubling the chromosome number of diploid material using colchicine and crossed the progeny to hexaploid oat. However, these transfers were genetically unstable and autotetraploids were partially sterile (Dyck and Zillinsky 1962; Sadanaga and Simons 1960). Marshall and Myers (1961) directly crossed *A. strigosa* with *A. sativa* and had no difficulty in obtaining seeds, but were only water filled and became extremely shrivelled when dry. Much work was done with CD 3820, an *A. strigosa* line, where several genes for crown rust resistance were found. Dyck and Zillinsky (1963) showed the presence of two independent genes, *Pc15* and *Pc23*. *Pc15* proved to be located on a chromosome that failed to pair with any *A. sativa* chromosomes. In lines homozygous for *Pc15*, the chromosome number was 44 where *Pc15* was found on the extra chromosome pair. *Pc23* appeared to be completely incorporated into normal *A. sativa* (Dyck and Zillinsky 1963).

Rines et al. (2007) attempted two methods to transfer crown rust resistance from a diploid *A. strigosa* into a hexaploid *A. sativa*. The first method directly crossed the diploid line CI6954SP with a hexaploid to obtain tetraploid F_1 progeny, which were subsequently treated with colchicine to generate a synthetic octaploid for subsequent backcrossing to the hexaploid parent. The second method crossed CI6954SP with a tetraploid *A. murphyi* line to make a synthetic hexaploid for subsequent crossing into a hexaploid background. Although the direct method requires the laborious crossing and embryo rescue to develop a fertile octaploid line, it provided faster recovery of plants with high fertility, full transmission of resistance and desired plant and seed phenotypes. Similar work was done by Zegeye (2008) to directly transfer stem rust resistance from *A. strigosa* into hexaploid oat. The general crossing scheme and methodology followed is shown in Fig. 3.1. Crosses and reciprocal crosses between the diploid *A. strigosa* accessions and hexaploid *A. sativa* cultivar Sun II were made, and embryo rescue and colchicine treatment produced octoploid progeny. Synthetic octaploid seeds were larger than the parental seeds (Fig. 3.2) and the seedlings were more vigorous. Progeny were selfed to the F_2 and F_3 generation and backcrossed to Sun II. Cytological evaluation of resistant BC_1F_2 progenies found 43–47 chromosomes (Fig. 3.3) and progeny tests of BC_1F_2 and BC_1F_3 with NA67 identified resistant seedlings. Rajhathy and Thomas (1974) reported that the line Cw57 can be used to induce chromosome pairing between the "A_s" genome of *A. strigosa* and the "A" genome of *A. sativa*. Thus, F_1 octaploid seeds were produced from crossing diploid *A. longiglumis* (Cw57) with Sun II, which was subsequently crossed to highly resistant BC_1F_3 progeny lines containing 43–44 chromosomes from CN57130/Sun II//Sun II. F_1 hybrid seeds were obtained, which will be repeatedly backcrossed to Sun II until the resistance from *A. strigosa* is stabilised in a 42-chromosome background.

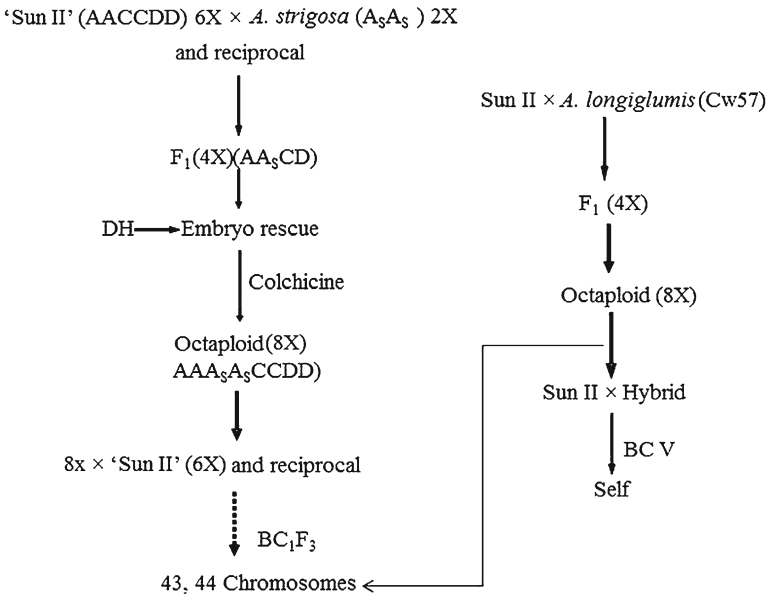

Fig. 3.1 Crossing scheme between diploid and hexaploid oat to transfer stem rust resistance from *A. strigosa* to *A. sativa*

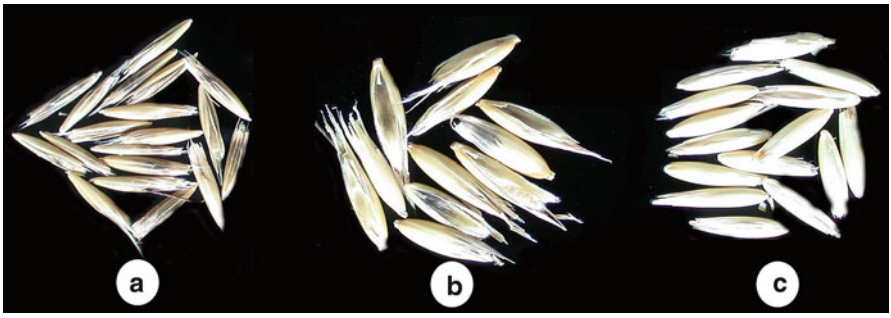

Fig. 3.2 Seeds from the diploid (**a**) *A. strigosa* (CN21997), (**b**) octaploid (CN21997/"Sun II") and (**c**) hexaploid *A. sativa* ("Sun II")

The use of irradiation to facilitate the transfer of resistance genes has also been reported. Mildew resistance was transferred from tetraploid *A. barbata* to cultivated oat *A. sativa* (Aung and Thomas 1976; Aung et al. 1977) using irradiation to induce translocations in a disomic addition line ($2n=6x=44$) of an *A. sativa* that contained a pair of *A. barbata* chromosomes carrying the mildew resistance gene. Sharma and Forsberg (1977) treated a monosomic substitution line with thermal neutrons and

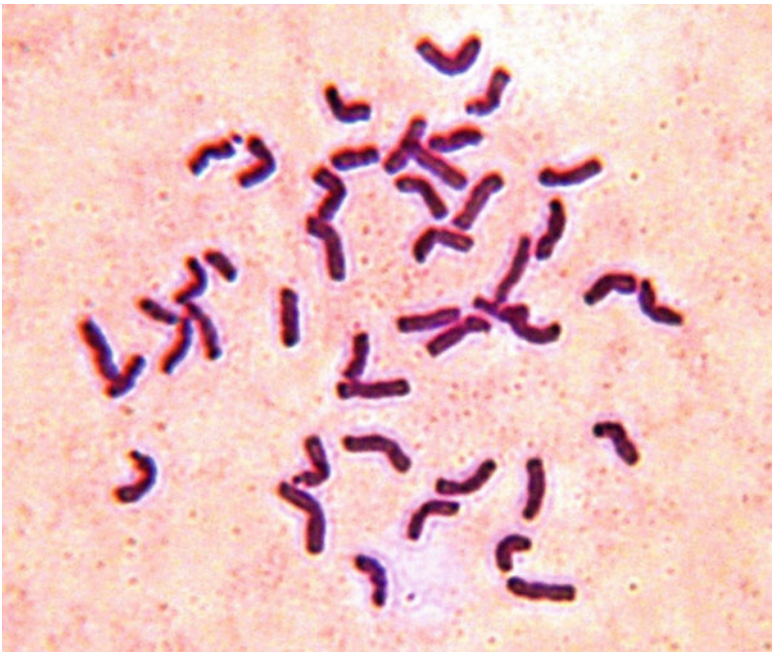

Fig. 3.3 Cytological evaluation depicting 44 chromosomes of a resistant BC_1F_2 seedling from CN21997/"Sun II"//"Sun II"

were successful in transferring the *Pc15* gene from *A. strigosa* into the cv. Clarian, which showed normal breeding behaviour and could be used in a breeding programme. The main limitation of the irradiation-induced gene transfers is that they are the products of reciprocal translocation and involve a deletion and duplication (Thomas 1992).

3.5 Relationship of Rust Resistance Genes and Molecular Markers

The exact position of rust resistance genes on oat chromosomes has not yet been described, because unlike the other cereal grains, there has not yet been any specific chromosome numbers allocated. Currently, there are 34 linkage groups mapped (Tanhuanpää et al. 2012), but work is underway to anchor molecular markers and develop a 21-chromosome map. Since no rust resistance genes have yet been located to a specific chromosome position, many genes could be alleles or the same gene since in most cases no full genetic studies exist. In crown rust, five linkage groups that have been identified and cultivars Santa Fe, Ukraine and Trispernia

have exhibited allelism or close linkage for at least one gene (Finkner 1954). A number of crown rust resistance genes are clustered in the oat genome, including *Pc46*, *Pc50* and *Pc68* (Wong et al. 1983); *Pc38*, *Pc62* and *Pc63* (Harder et al. 1980); *Pc39* and *Pc55* (Kiehn et al. 1976); *Pc35*, *Pc54* and *Pc96* (Martens et al. 1980; Chong and Brown 1996); and *Pc68*, *Pc44*, *Pc46*, *Pc50*, *Pc95* and *PcX* (Chong et al. 1994). Leonard et al. (2005a) suggested that genes *Pc39*, *Pc55* and *Pc71* may be identical or nearly identical alleles. It has also been postulated that gene *Pc94* might be close to or part of *PcA* (Rines et al. 2007). In stem rust, genes *Pg1*, *Pg2*and *Pg8* are clustered, *Pg3* and *Pg9* are linked, and *Pg4* and *Pg13* are associated (Martens 1985).

Crown rust and stem rust resistance genes have also been shown to be linked together. Martens et al. (1968) suggested that there were three alleles for crown rust resistance and two alleles for stem rust resistance at or near the *Pc44* locus, and *Pc44* was linked in repulsion to *Pg9*. *Pg9* was associated with crown rust resistance in Ukraine oat (McKenzie et al. 1965). The mapping of resistance gene analogues (RGA) on the Kanota × Ogle (KO) by Sanz et al. (2012) showed that both the nucleotide binding site (NBS) and protein kinase (PK)-based markers significantly co-localised with loci conferring resistance to *P. coronata*, i.e. *Pc39* (KO16_23), *Pc54*/*Pc59* and *Pc68* (KO4_12) and *Pc58* (KO17), as well as with a locus conferring resistance to *P. graminis*, i.e., *Pg13* (KO3 + 38).

Many race-specific resistance genes have been mapped, and markers that are closely linked to crown and stem rust genes have been identified (Table 3.3). An *avenin* protein marker is linked to *Pg3* and *Pg9* (Howes et al. 1992; Chong et al. 1994), and three *avenin* storage protein loci as well as two RGA markers are very tightly linked to *Pc68* (Satheeskumar et al. 2011). A random amplified polymorphic DNA (RAPD) marker has been linked in repulsion to *Pg3* (Penner et al. 1993a). The latter RAPD primers also produced a marker which, together with three restriction fragment length polymorphism (RFLP) markers and a third RAPD marker, were shown to be linked to *Pg9* (O'Donoughue et al. 1996). Gene *Pg13* has been mapped in two populations to linkage groups homologous to KO3 (O'Donoughue et al. 1996) and a second stem rust resistance gene *Pg4* (McKenzie et al. 1970). *Pg13* is also linked to a 56.6-kDa avenin storage protein marker (Howes et al. 1992; Chong et al. 1994). Personal Communication (2013) developed SCAR, CAPS and SSR markers based on amplified fragments linked to *Pg3*, *Pg9* and *Pg13* obtained using the RAPD primers ubc195, ubc269 and ubc254. Molecular markers can be used to facilitate pyramiding of genes, a breeding strategy designed to provide more durable control of rust by combining several resistance genes in one cultivar (Pedersen and Leath 1988).

Molecular markers can also be used in counter-selection. The gene *Pc38* would be an excellent choice, as it is known to suppress the action of genes *Pc62* (Wilson and McMullen 1997) and *Pc94* (Chong and Aung 1996). Molecular markers developed for any one particular rust resistance gene in a cluster will also be useful for the study of other disease resistance genes found within the same cluster. A KASP assay was developed from the previously reported nonredundant DArTs that co-segregated with *Pc91* (Gnanesh et al. 2013). The KASP assay was used for marker-assisted

Table 3.3 Molecular markers for oat crown and stem rust resistance genes

Gene	Marker	Linked marker/QTL name	Reference
Crown rust			
Pc38	RFLP	cdo673, wg420	Wight et al. (2004)
Pc39	RFLP	cdo666	Wight et al. (2004)
Pc48	RFLP	cdo337	Wight et al. (2004)
Pc54	RFLP	cdol435B	Bush and Wise (1996)
Pc58a,b,c	RFLP	PSR637, RZ516D	Hoffman et al. (2006)
Pc59	RFLP	cdo549B	Bush and Wise (1996)
Pc68	RAPD	ubc269	Penner et al. (1993b)
	SNP	*Pc68*-SNP1, *Pc68*-SNP2	Chen et al. (2006)
	AFLP	U8PM22, U8PM25	Kulcheski et al. (2010)
	SDS-PAGE	AveX, AveY, AveZ	Satheeskumar et al. (2011)
	RGA/RFLP	Orga1	Satheeskumar et al. (2011)
	SCAR	ubc269s SCAR	Personal Communication (2013)
Pc71	RFLP	cdo783, cdo1502	Bush and Wise (1998)
Pc81,82, 83,84,85	AFLP	isu2192, OP C18	Yu and Wise (2000)
	STS	Agx4, Agx9, Agx7	Yu and Wise (2000)
Pc91	RFLP	UMN145	Rooney et al. (1994)
	DArT	oPT-0350	McCartney et al. (2011)
	SCAR	oPT-0350-cdc	McCartney et al. (2011)
	KASP	oPT-0350-KOM4c2	Gnanesh et al. (2013)
Pc92	RFLP	OG 176	Rooney et al. (1994)
Pc94	AFLP	AF94a	Chong et al. (2004)
	SCAR	SCAR94-1, SCAR94-2	Chong et al. (2004)
	SNP	*Pc94*-SNP1a	Chen et al. (2007)
Pca	RGA/RFLP	isu2192	Kremer et al. (2001)
		L7M2.2	Irigoyen et al. (2004)
		b9-1	Sanz et al. (2012)
PcX	RFLP, RAPD	Xcdo1385F, XpOP6(A), Xacor458A	O'Donoughue et al. (1996)
Stem rust			
Pg3	RAPD	ACOpR-1, ACOpR-2	Penner et al. (1993a)
	SCAR/CAPS	*Pg3* SCAR/CAPS	Personal Communication (2013)
Pg4	SCAR/CAPS	ubc254s SCAR	Personal Communication (2013)
Pg9	Acid-PAGE	*avenin* band	Chong et al. (1994)
	RFLP, RAPD	Xcdo1385F, Xacor458A	O'Donoughue et al. (1996)
	SCAR/CAPS	*Pg9* SCAR/CAPS	Personal Communication (2013)
Pg13	SDS-PAGE	56.6-kDa polypeptide locus	Howes et al. (1992)
	RFLP, RAPD	Xmog12B, Xacor254C	O'Donoughue et al. (1996)
	SCAR	*Pg13* SCAR	Personal Communication (2013)
Sr_57130	AFLP	PacgMcga370	Zegeye (2008)

selection for crown rust resistance gene *Pc91* in an F_2 population developed from the cross of AC Morgan × Stainless (Fig. 3.4). Sanz et al. (2012) reported the PK-RFLP and PK profiling markers co-localise with *Pc71* (KO11_41 + 20) and *Pc91* (KO3 + 38), whereas clusters of mixed markers based on NBS and PK domains

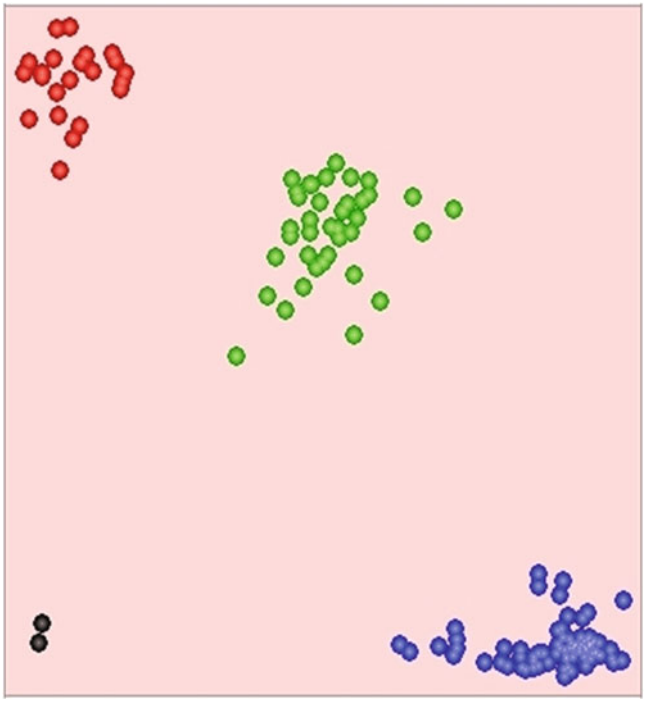

Fig. 3.4 Marker-assisted selection for crown rust resistance gene *Pc91* using oPt-0350-KOM4c2 KASP SNP assay in F_2 population (AC Morgan × Stainless). *Red* data points are carriers of *Pc91*, *blue* data points are non-carriers, *green* data points are segregating and *black* data points are no template controls

co-localise with genes *Pc38* (KO17), *Pc94* (KO17) and *PcX* (KO4_12). Comparative mapping of disease resistance loci with reference populations such as Kanota × Ogle (KO) (Wight et al. 2003), Ogle × TAM O-301 (Portyanko et al. 2001) and Ogle × MAM17-5 (OM) (Zhu and Kaeppler 2003) increases the number of potential molecular markers available for resistance genes and furthers our understanding of their organisation in the genome. The development of anchored markers on a 21-chromosome map is the first step in identifying the location of rust resistance genes and would help immensely in the identification of new resistance genes introgressed from wild species.

3.6 Future Outlook on Alien Introgression in Oat

Wild species are an important and abundant source of new rust resistance genes. Previous studies have identified numerous rust resistance genes in diploid and tetraploid wild species, but few have been successfully transferred. Although it

appears that initial transfer into chromosome addition lines is relatively simple, introgression and reduction to 42-chromosome cultivated oat have been difficult. New techniques are needed to find better methods of stabilising rust resistance from wild species, as virulence in *P. coronata* and *P. graminis* f. sp. *avenae* appears to be increasing worldwide.

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Chapter 4
Pearl Millet

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Abstract With an alarming concern of global climate change and increasing demand for drought-tolerant cereal staples, pearl millet, a widely exo-geographically adopted member of the millet family, has caught the attention for a robust genetic improvement. This chapter focuses on the various gene transfer technologies, both horizontal and vertical, employed so far in pearl millet to improve the strategy of introgression of newer traits and validation of gene function through transgenic development. This chapter also compares the different gene transfer technologies based on their exploitation in pearl millet development. It also accounts for the details of genes transferred so far, especially for conferring biotic and abiotic stress tolerance, in this crop. This chapter also discusses the future possibilities regarding the introgression of genes of new traits and technologies already utilized in other millets, which are hitherto unexploited for pearl millet.

Keywords Abiotic stress • *Agrobacterium*-mediated transformation • Biolistics • Biotic stress • Gene transfer • Pearl millet

4.1 Introduction

Millets, which rank as the world's sixth most important tropical food cereal (see FAOSTAT at www.fao.org), are grown mostly in the semi arid West Africa and India. Pearl millet is the only major cereal that reliably produces both grain and forage on poor, sandy soils under hot and dry conditions. In the drier regions of Africa and Asia, this crop is a staple food grain, besides being extensively utilized as a summer

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annual grazing crop in the southern USA (Hanna et al. 1997). The release of new high-quality forage and grain cultivars, combined with pearl millet's natural drought resistance, is gradually increasing the US commercial growers' interest in this underexploited, multipurpose crop.

Millet crops are grasses that belong to the family Poaceae of the monocotyledon group. Millets are staple foods that supply a major proportion of calories and protein to large segments of populations in the semiarid tropical regions of Africa and Asia (O'Kennedy et al. 2006). The semiarid tropics are characterized by unpredictable weather, limited and erratic rainfall, and nutrient-poor soils and suffer from a host of agricultural constraints (Sharma and Ortiz 2000). The projected food demand for 2025 will require the yield of cereals, including millets, to rise from 2.5 to 4.5 t/ha (Borlaug 2002). Production of millets, besides abiotic stresses, is also constrained by fungal diseases; so host plant resistance through alien gene transfer as well as genetic engineering is essential for conferring high level of resistance to improve the yield of millets (Ceasar and Ignacimuthu 2008).

4.2 Taxonomy and Gene-Pool Concept

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is a highly cross-pollinated monocot belonging to the Poaceae. It is one of the most widely cultivated drought- and high-temperature tolerant C4 cereals, being grown for forage, grain, and stover under dryland, rainfed, and irrigated conditions in drought-prone regions of the arid and semiarid tropics and subtropics and as a mulch in conservation tillage production systems in the humid and subhumid tropics. It is especially important as a staple food grain and source of feed and fodder for livestock, in hot, dry marginal agricultural production environments of Africa and South Asia that are home to hundreds of millions of the world's poorest farmers. The genus *Pennisetum* consists of more than 140 species of various ploidy levels that display annual and perennial life cycles and sexual, asexual, and apomictic reproductive behavior (Hanna 1987). Sexual reproduction through outbreeding is the most common mode of reproduction in cultivated pearl millet and its wild and weedy relatives. Harlan and de Wet (1971) classified *Pennisetum* germplasm in to three gene pools on the basis of cross-ability relationships and following the biological concept of species. They reported that the primary gene pool consists of all the cultivated forms, their wild progenitor (*Pennisetum glaucum* ssp. *violaceum* (= *monodii* Maire)), and its weedy form (*Pennisetum glaucum* ssp. *stenostachyum* Kloyasch ex. A. Br., and Bouche). Species belonging to this gene pool are all diploid with $2n = 14$. Secondary gene pool comprises of only elephant or Napier grass (*Pennisetum purpureum* Schumach.), which is an allotetraploid ($2n = 4x = 28$) and rhizomatous perennial. All other species are part of the tertiary gene pool.

The classical botanists have tried to establish the genetic relationships between wild and cultivated pearl millets. Based on crossing of two wild × cultivated crosses, annual wild and cultivated species have been grouped into a single biological species (Bilqueza and Lecomte 1969). Other study based on the excellent fertility

observed in hybrids derived from three wild × cultivated crosses also concluded these species as a single biological species but with three botanical subspecies corresponding to cultivated, wild, and intermediate types (Brunken et al. 1977). However, cultivated pearl millet has been classified into four basic races (*typhoides*, *nigritarum*, *globosum*, and *leonis*) on the basis of seed shape (Brunken et al. 1977). In subsequent studies, another series of hybrids (wild × cultivated) were studied, which did not show any divisions within the biological species (Belliard et al. 1980; Pernes et al. 1980). These authors defined it as a "domestication syndrome" which refers to the type of genetic organization of the characters differentiating wild and cultivated genotypes.

4.3 Genetic Resources

The International Crops Research Institute for the Semiarid Tropics (ICRISAT) is the only major repository of *Pennisetum* germplasm and has 21,191 accessions from 49 countries, including 20,503 accessions of cultivated types, 334 accessions of ssp. *monodii*, and 354 accessions of 22 other species. This collection is from diverse geographical areas including Africa, stretching from South Africa to Somalia in the east and Senegal in the west and Asia. In addition to this, few other centers also hold sizeable *Pennisetum* germplasm. Among these, there are three gene banks in the USA with a total of 6,637 accessions (including 805 accessions of 31 wild relatives) (Hanna and Lovell 1995), ORSTOM (Institut Français de Recherche Scientifique pour le Développement en Coopération) at Bondy in France (2,700 accessions), and ISRA at Bambey in Senegal (2,400 accessions) (Rai et al. 1997). Western Africa is the primary center of diversity for *Pennisetum*.

4.4 Vertical Alien Gene Transfer Using Cross-Compatibility

4.4.1 Conventional Approach

The utilization of wild germplasm for transferring the desirable alien genes has not been used widely owing to problems in cross-compatibility. Pearl millet has been hybridized with a number of wild species, including *P. orientale* L. C. Rich (Hanna and Dujardinm 1982), *P. schweinfurthii* Pilger (Hanna and Dujardinm 1986), and *P. setaceum* Forsk. Chiov. (Hanna 1979). Subsequently, cross-compatibility between diploid and tetraploid species was studied in detail (Dujardin and Hanna 1989). They reported that interspecific hybrids between pearl millet and *P. ramosum*, *P. mezianum*, *P. macrourum*, *P. pedicellatum*, and *P. polystachion* showed cross-incompatibility. However, partial seed development in diploid pearl millet × *P. pedicellatum* or *P. polystachion* crosses indicated possibilities of using embryo culture technique for recovering interspecific hybrids. Though wild species *P. setaceum* showed cross-compatibility with pearl millet, this culminates in a dead end for the

breeders because of complete male sterility and poor female fertility with obligate apomixis. The use of *P. orientale* has also been limited for alien gene transfer due to lack of recombination between the genomes of the two species (Dujardin and Hanna 1989). The wild species *P. squamulatum* has been identified as a valuable species for transfer of important traits to pearl millet due to its high frequency as well as the high level of male fertility. In general, species with $x=9$ of the tertiary gene pool (*P. setaceum*, *P. orientale*, and *P. squamulatum*) have been shown more readily crossing with pearl millet than the $x=5$ (*P. ramosum*) and $x=8$ (*P. mezianum*) species (Dujardin and Hanna 1989). Transfer of alien genes for rust resistance to pearl millet has been accomplished from a wild subspecies *P. glaucum* (L.) R. Br. ssp. *monodii* (Maire) due to chromosome homology (Hanna et al. 1985). Alien genes controlling earliness, long inflorescences, leaf size, and male fertility restoration have also been transferred from Napier grass, *P. purpureum* Schumach. (a wild alloploid species) for improving pearl millet. It could be possible due to one genome similarity with the genome of pearl millet (Hanna 1983).

Few efforts have been made at Tifton, Georgia, for using accessions of wild species, namely, *Pennisetum glaucum* ssp. *monodii* for rust and leaf spot resistance and cytoplasmic male sterility, *P. Purpureum* for greater stalk strength and male fertility restorer genes, and *Pennisetum squamulatum* for apomictic genes (Hanna 1992). More recently, the wild grassy subspecies *Pennisetum glaucum* (L.) R. Br. ssp. *monodii* (Maire) Brunken has been used as a source of germplasm for improved disease resistance and cytoplasmic diversity for improving pearl millet [*P. glaucum* (L.) R. Br.] cultivars. The hybrids for forage produced by crossing between the accessions of wild-species *monodii* from Niger, Mali, Senegal, and Bur-kina Faso and Tift 85DA1, a cytoplasmic-nuclear-male-sterile (CMS) pearl millet, showed significant increase in dry matter. This research indicates that alien genes from wild grassy subspecies *monodii* have enhanced the level and distribution of yield in cultivated pearl millet (Hanna 2000). The completely male-sterile lines were also produced in the wild cytoplasm background (*Violaceitm* and *P. mollissimttm*). These were produced following the backcross method and resulted in inbred lines that resembled greatly to the cultivated parent. Restoration tests of this new male-sterile line indicate that it is different from the three known male-sterile sources Al, A2, and A3. Wild forms seem to possess a higher frequency of restorer alleles than the cultivated forms, regardless of their geographical origins (Marchais and Pernes 1985). Genetic diversity based on new molecular tools showed differences between the cultivated and wild groups in Niger. This study also showed introgression of cultivated alleles into wild accessions in the central region of Niger, while wild alleles were introgressed in cultivated species in the western, central, and eastern parts of Niger (Mariac et al. 2006).

4.4.2 Marker-Based Vertical Gene Transfer

In recent years, molecular marker-based alien gene transfer from wild species is being widely utilized in crop plants. In pear millet, molecular markers have been

used to identify the genes for abiotic stress, and efforts were made to transfer such genes into cultivated background. Moisture stress is one of the important abiotic stresses that affects at various growth stages, but yield losses are maximum when moisture stress coincides with grain filling stage (i.e., terminal water stress). Genetic differences in tolerance to salinity and high temperature at both seedling and grain filling stages have been established and screening techniques standardized (Yadav et al. 2010). Use of conventional approaches to improve drought tolerance in pearl millet has been difficult despite the use of some novel approaches such as use of adapted germplasm, genetic diversification of adapted landraces through introgression of suitable elite genetic material, and exploitation of heterosis to amalgamate drought tolerance and high yield for enhancing yield under drought environments.

Molecular marker-based genetic linkage maps of pearl millet are available and genomic regions determining yield under drought environments have been identified. These efforts have paved the way for precise introgression of drought-tolerance-related traits through marker-assisted selection. The first molecular marker-based genetic linkage map of pearl millet, comprising largely of RFLP loci supplemented by a few isozyme loci, was reported by Liu et al.(1994). In subsequent years, the linkage map was expanded with SSR markers (Qi et al. 2004) and DArT markers (Supriya et al. 2011). A number of studies on quantitative trait loci (QTLs) for drought tolerance (Yadav et al. 2002, 2004), components of drought adaptation (Kholova et al. 2012), flowering time, and grain and stover yield (Yadav et al. 2003) have been mapped, and effective marker-assisted selection for several of these traits has been demonstrated (Serraj et al. 2005; Hash et al. 2003). These studies clearly showed that molecular markers can also be used to map the genes in the background of wild species, which can be precisely introgressed in cultivated background through marker-based selection (Rajaram et al. 2013).

4.5 Horizontal Alien Gene Transfer

Traditional breeding has been the main avenue for crop improvement in pearl millet. However, genetic transformation is a valuable tool useful to transfer alien genes for resistance to diseases, insects, and herbicides from those sources which are inaccessible through traditional plant breeding approaches. Using this approach several cereal crops have been transformed (Repellin et al. 2001). This approach can be used to test the effect of putative apomixes gene(s) from a wild relative (*Pennisetum squamulatum*; Ozias-Akins et al. 1998).

4.5.1 Biotic Stress

Fungal diseases are a major constraint in crop production imparting high yield loss. Significant yield losses occur in most of the agricultural and horticultural species due to fungal attacks. In Indian context, fungal diseases are rated either the most

important or the second most important factor contributing to yield losses in major cereal, pulse, and oilseed crops (Grover and Gowthaman 2003). Several successful attempts have been made to develop fungus-resistant cereal crops through genetic engineering. The chitinase gene has been considered best candidate for defending crop plants from fungal diseases. The first transgenic pearl millet expressing functionally active foreign gene conferring resistance to fungal disease (downy mildew) has been produced by Girgi et al. (2006). They used the antifungal protein (*afp*) gene isolated from the ascomycete, *Aspergillus giganteus*, using immature zygotic embryos as target for biolistic transformation. Transformation of pearl millet was confirmed by molecular analysis; the disease resistance also increased up to 90 % when compared to non-transformed control plants. Latha et al. (2006) have also developed a transgenic pearl millet conferring resistance to downy mildew disease by inserting a chemically synthesized prawn antifungal protein encoding gene (*pin*); embryogenic calli were used as target explants for bombardment. However, only two fertile transgenic pearl millets expressing functional foreign genes have been reported so far (Girgi et al. 2006; Latha et al. 2006). Chitinase and glucanase genes have also been shown promising against fungal pathogens. Hence, in the near future, these alien genes could be transferred in pearl millet in order to offer the prospect of conferring high levels of resistance against fungal pathogens and thereby improving pearl millet production.

4.5.2 Abiotic Stress

Significant genetic diversity exists among plant species for stress adaptation, hence elucidating the transcriptome from abiotic stress-adapted species. Several reports have shown that genes from stress-adapted species are functionally more efficient in imparting tolerance (Bartels and Salamini 2001; Whittaker et al. 2001; Mundree et al. 2002). Stress-adapted species and resurrection plants with very high tolerance threshold may possess mechanisms and genes which make them survive extreme conditions. A few recent studies have been initiated to understand the drought-tolerant traits in terms of identification of QTLs in pearl millet, exploiting the available pearl millet EST sequences to generate a mapped resource of 75 new gene-based markers for pearl millet. These demonstrated its use in identifying candidate genes underlying a major DT-QTL in this species. The reported gene-based markers represent an important resource for identification of candidate genes for other mapped abiotic stress QTLs in pearl millet (Sehgal et al. 2012). They also provide a resource for initiating association studies using candidate genes and also for comparing the structure and function of distantly related plant genomes such as other Poaceae members. Also, these candidate genes can be used for thoroughly elucidating its role in drought tolerance in other species and to develop transgenics. Being an abiotic stress-tolerant crop, it is of not much relevance to transfer any other stress gene homologue from allied species into millets. On contrary, the abiotic stress tolerance genes of millets, although very little characterized and reported, are of great importance for enhancing stress tolerance in other plant species—indeed, a good example of bioprospecting.

4.6 Conclusions and Future Prospects

The wild gene pool contains important genes that might be transferred to cultivated pearl millet and utilized in creating useful genotypes. However, interspecific hybridization requires great efforts depending upon the relationships of the species crossed. In general, use of wild species for transferring the alien genes has not been accomplished widely in pearl millet as compared to other cereal crops. Therefore, more efforts are required to study species relationships and utilization of many different accessions of many species. This can result in identification of readily crossable accessions of wild species. The efforts can also be made on attempting many crosses before producing one or a few interspecific hybrids.

Genetic transformation is an important approach that can be used to transfer alien gene from wild species as well as other sources. Therefore, efforts made in the *Agrobacterium*-mediated transformation of other cereals need to be extended to millets in the near future to produce transgenic millets expressing agronomically important foreign genes. This will greatly help to improve millet production by conferring resistance to biotic and abiotic stresses.

Conservation of phenological diversity is an important aspect for the future pearl millet improvement and sustainable use. Orthologous genes have been identified in pearl millet for flowering on the basis of candidate gene-based approach. This approach can also be extended to identify the agronomically important genes in the background of cross-incompatible wild species. It well known that future of pearl millet improvement through genetic engineering largely depends upon discovery of novel genes attributing the potent water deficit stress tolerance property of this important crop. As the recent addition of millet improvement, the foxtail genome analysis discovered a family of 586 genes functionally annotated as "response to water," which further indicates that foxtail millet-specific genes might be related to adaptation of foxtail millet to semiarid environments. Thus, identification and isolation of alien genes/novel genes through recent genomics tools and techniques can help to use them in pearl millet improvement using transgenic approach (Lakis et al. 2012).

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Chapter 5
Barley

Jutta Schulze

Abstract Barley (*Hordeum vulgare* L.) is a widely adapted cereal crop with an extremely wide geographic distribution throughout the world. It finds great use for animals as a feed and for humans as a grain, especially as the source for malt for the brewing industry. In recent times, there is considerable interest in the nutritional properties of barley due to the discovery of the cholesterol-lowering effect of β-glucan, a cell wall polysaccharide. Exploitation of genetic diversity in the primary and secondary gene pool of barley using DNA-based technologies has yielded interspecific crosses with improved grain properties, malting quality and resistance to biotic and abiotic stresses. The significant achievements regarding introgression of alien genes include the genes *Rym14(Hb)*, *Rym16(Hb)* and *Ryd4(Hb)* which were introgressed from *Hordeum bulbosum* conferring resistance to BaMMV, BaYMV and BYDV in barley. Significant advances in genetic engineering of barley have been obtained, and strategies for establishment of regenerative cell and tissue culture systems as well as for development of DNA delivery techniques have been formulated. Lately, a huge potential has been realised in barley grains to produce pharmaceutical proteins like oral vaccines, growth supplements and food additives which are being exploited in a commercial scale. Nevertheless, several problems still remain like the strong genotype dependency of barley transformation protocols, transformation efficiency, transgene stability and public acceptance. The review focuses on all these issues and elaborates achievements made in the last two decades in genetic enhancement of barley using different alien gene transfer approaches.

Keywords Abiotic stress • Barley • Biopharming • Disease resistance • Field trials • Gene transfer • Wide hybridisation

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5.1 Introduction

Crop plants derived from their ancestors in a long process from preferential harvest of wild plants to directed selection of plants with good performance resulted in systematic breeding for desirable agronomic traits. The process, which started approximately 10,000 years ago along with settlement of men, resulted in high yielding cultivars to meet the demands of the ever-increasing population of the world. However, elite cultivars are vulnerable to a wide range of pressures, thus plant breeders must constantly respond to adapt and improve crops.

More than half of the food consumed by mankind is based on the major cereals, viz. maize, wheat, rice and barley, since these crops are the main sources of plant carbohydrates and proteins (FAO 2012). Thus, cereals are substantial for production of animal feed, starch, flour, sugar, oils, processed foods, malt, alcoholic beverages, gluten and renewable energy (Edgerton 2009). The "Green Revolution" and intensification of crop management led to an increase in productivity of these crops until the 1980s (Hedden 2003). However, in the last two decades, growth rates of yields slowed down due to declining resources of arable land and water, deteriorating soil conditions as a result of environmental degradation and climate change (Schmidhuber and Tubiello 2007; Mba et al. 2012) as well as due to limitations in the germplasm pool (McIntosh 1998; Prada 2009). It is estimated that feeding nine billion people in 2030 would demand raising overall food production by some 50 % between 2005/2007 and 2030 (Beddington 2010; Wegner and Zwart 2011). Thus, there is an urgent need for new approaches and technologies and also for generating new varieties to meet that dramatic increase (Edgerton 2009; Phillips 2010).

Barley (*Hordeum vulgare* L.), the number four in the world's cereal crops with respect to production quantity, yield (t/ha) and acreage (FAOSTAT), is a widely adapted plant with an extremely wide geographic distribution. In 2011, the estimated world production of barley was 134 million tons (FAOSTAT). The largest use is for animal as well as human food, especially as the source for malt for the brewing industry. However, in recent time there is considerable interest in the nutritional properties of barley due to the discovery of the cholesterol-lowering effect of β-glucan, a cell wall polysaccharide found in barley (Newton et al. 2011).

H. vulgare is well studied regarding genetics, cytogenetics and genomics. Cultivated barley is self-pollinated and diploid with $2n = 2x = 14$ chromosomes, and its genome size is about 5.5×10^9 bp with 80 % highly repetitive DNA. Besides the availability of numerous germplasm collections, a lot of data have been accumulated in recent time concerning molecular markers, genomic DNA sequences, full-length cDNAs and expressed sequence tags (ESTs) supplemented by voluminous studies on genomics, proteomics and metabolomics which are accessible in different databases (Sreenivasulu et al. 2008). Strikingly, sequencing the genome of barley has become a realistic task (Schulte et al. 2009). In parallel, tremendous efforts in cereal transformation technology were made allowing now comprehensive functional analysis of genes. Combining these developments, barley is now a model plant for the *Triticeae* (Saisho and Takeda 2011), which is reflected by numerous

reports on transgenic barley in very recent time. This chapter attempts to summarise achievements made in alien gene transfer in barley with emphasis on agronomic traits as well as fundamental research.

5.2 Introgression of Alien Genes by Wide Hybridisation

A key to successful barley protection and high yield is the constant genetic improvement in this crop. Barley breeding has a long and prosperous history with respect to enhancement in resistance levels and yield, and extensive progress has been achieved in the last few decades (Friedt et al. 2011). However, the modern elite cultivars show a relatively low level of genetic diversity, and the loss of important traits like resistances have led to genetic uniformity due to gene erosion. Nevertheless, barley is challenged by a range of biotic and abiotic stresses, and continuously, (1) new sources emerged due to climate change and (2) the pathogens respond with a rapid adaptation (Gregory et al. 2009; Pautasso et al. 2012). The traditional way for introgression of new genes or for the combination of desired traits is sexual recombination combined with phenotypic selection and analysis of the progeny. However, classical breeding procedures are seriously challenged if sources of natural resistance to pathogens are rare as in case of barley yellow dwarf virus (BYDV) (Ordon et al. 2005; Kosová et al. 2008).

5.2.1 Wide Crosses

Wild or related species of cultivated crops represent large resources for desirable genes. In attempts to exploit a broader germplasm resource for improvement of barley, wild relatives and landraces were re-evaluated (Pickering and Johnston 2005; Steffenson et al. 2007; Newton et al. 2010; Nevo and Chen 2010). Various aspects of introgressing genes from wild barley into domesticated barley have been comprehensively covered previously (Fedak 1989). The ancestor of domesticated barley, *H. vulgare* ssp. *spontaneum*, which belongs to the primary gene pool, shows no incompatibility in crossings, while hybridisation with *Hordeum bulbosum*, the only member of the secondary gene pool, is difficult (von Bothmer et al. 2003). The use of these wild relatives as a source in breeding programmes can be realised by using the embryo rescue technique. However, strong crossability barriers exist to the 30 species of *Hordeum* belonging to the tertiary gene pool (Pickering and Johnston 2005). Successful reports on transfer of resistances against several threats like powdery mildew (Pickering et al. 1995), leaf rust (Pickering et al. 1998), Septoria speckled leaf blotch (Toubia-Rahme et al. 2003) and barley yellow mosaic virus (Ruge et al. 2003; Ruge-Wehling et al. 2006) from *H. bulbosum* into *H. vulgare* are available.

Recently, the development of a set of introgression lines (Ils) for barley was reported with each IL carrying a single introgression of the exotic *H. vulgare* ssp.

spontaneum accession ISR42-8 in the genetic background of the elite spring barley cultivar Scarlett. The set was generated by backcrossing, selfing and marker-assisted selection. In order to illustrate the applicability of the spring barley ILs, the lines were used for verification of quantitative trait loci (QTLs) for field resistance against powdery mildew (*Blumeria graminis* f. sp. *hordei* L.) and leaf rust (*Puccinia hordei* L.) (Schmalenbach et al. 2008). In addition, QTLs were verified in these ILs affecting malting quality parameters (Schmalenbach and Pillen 2009). It was validated that wild barley alleles at the chromosome 1H QTL reduced overall malting quality, whereas wild barley alleles at the chromosome 4H QTL improved the malting quality parameters compared to the control genotype Scarlett (March et al. 2012). Furthermore, a set of 110 putative ILs containing chromatin introgressed from *H. bulbosum* L. into cultivated barley has been identified using a high-copy number retrotransposon-like PCR marker. Introgressed chromatin from *H. bulbosum* was confirmed and genetically located in 88 of these lines using 46 of the EST-derived PCR markers (Johnston et al. 2009). Notably, resistance to stem rust, caused by *Puccinia graminis* f. sp. *tritici*, which is an important disease in *H. vulgare*, was transferred from *H. bulbosum* into cultivated barley (Fetch et al. 2009). Moreover, the successful transfer of *Ryd4Hb*, a novel resistance gene introgressed from *H. bulbosum* into barley and conferring complete and dominant resistance to the barley yellow dwarf virus, has to be highlighted (Scholz et al. 2009).

5.2.2 Somatic Hybridisation

An alternative approach to transfer genes from distant species or from wild relatives is hybridisation of somatic cells to circumvent sexual incompatibilities. Despite a large number of somatic hybrids that have been produced in a number of crop plants (Liu et al. 2005), there are only a very few reports on protoplast fusion in barley. Application of this technique for barley improvement is severely limited due to difficulties encountered with plant regeneration from barley protoplasts as will be discussed later. The formation of hybrid calli between *H. vulgare* L. and *H. bulbosum* was described, but plant regeneration was not reported (Funatsuki et al. 1994).

Protoplast fusion experiments in barley and rice yielded one intergeneric somatic hybrid plant, which resembled with rice in its morphology. Cytological studies revealed large chromosomes from barley and small chromosomes from rice. Southern hybridisation with a fragment of the *tryptophan B* (*trpB*) gene detected barley-specific and rice-specific bands. Furthermore, novel mitochondrial and chloroplast sequence rearrangements were also reported that were not detected in either of the parents (Kisaka et al. 1998). Fusion between barley and carrot protoplast was also carried out in an attempt to transfer cold and salt tolerance from barley into carrot. Morphology of the three regenerated plants closely resembled that of the parental carrot plants (Kisaka et al. 1997).

5.3 Application of Biotechnological Approaches

Biotechnological methods can improve the efficiency of barley breeding since (1) they offer the opportunity to access to additional gene pools, (2) allow the insertion of individual genes and (3) reduce time-consuming conventional techniques of crossing and backcrossing. Transfer of genes, which cannot be introduced via sexual hybridisation due to pre- and post-zygotic incompatibilities, requires gametic or somatic cells competent for regeneration. Thus, one prerequisite for the production of fertile transgenic plants is a totipotent target tissue and, second, methods to deliver DNA into these cells. Both the processes, which have been developed independently, have to be combined to provide highly efficient, cost-effective and easy to handle protocols as a powerful tool for crop improvement programmes and for analysis of gene function.

5.3.1 Establishment of Regenerative Systems in Barley

With respect to cell biology, single cells like protoplasts are an ideal target for direct DNA uptake. As described for the other cereals, protoplasts in barley can be isolated in large quantities from the leaves, roots and stems. Nevertheless, only in rare and irreproducible cases protoplasts divided to form callus. Thus, rapidly growing embryogenic cell suspensions were used as alternative source, but plant regeneration as a prerequisite for generating transgenic plants could not established as a routine method (Jähne et al. 1991; Funatsuki et al. 1992; Davey et al. 2005).

In parallel, multicellular explants were analysed to establish highly regenerative systems. Leaves derived from in vitro as well as ex vitro grown plants, which are the preferential source for callus initiation with subsequent plant regeneration in dicots, gave only a poor or no response in barley as well as for all other members of the cereals. Thus, other explants like mature seeds, isolated mature embryos, tissues derived from young seedlings, immature embryos, inflorescences, nodes and roots were evaluated, and a large number of reports are available (reviewed in Schulze 2007). In the second half of the 1980s, a general consent emerged that immature embryos are the most suitable explants. Additionally, anthers and microspores, extensively analysed for the production of haploid barley plants, reveal embryogenic capacity thus being an excellent target for gene transfer since homozygous transgenic plants can be developed more rapidly (Devaux and Kasha 2009).

Nevertheless, irrespective of the explant source used, the regeneration potential in barley is strongly affected by several factors like genotype, medium composition, phytohormones and growing conditions of the donor plants. There are a number of reports, which demonstrate that tissue culture ability and green plant regeneration is under genetic control (Bregitzer and Campbell 2001; Tyankova and Zagorska 2001). Further, an ever-increasing number of reports deal with improvements of regenerability by optimising or adapting media components for elite

genotypes. Consequently, some progress has been made, for example, by substitution of 2,4-D by picloram (Przetakiewicz et al. 2003; Chauhan and Kothari 2004) or dicamba (Halámková et al. 2004; Aguado-Santacruz et al. 2011), supplement of thidiazuron (Schulze 2007; Gubisová et al. 2012), increasing cupric sulphate (Bregitzer et al. 1998a; Nuutila et al. 2000) or incorporating ethylene precursor 1-aminocyclopropane 1-carboxylic acid or adding ethylene antagonist silver nitrate (Jha et al. 2007; Tyagi and Dahleen 2011). However, these manipulations also could not affect the genotype dependency with respect to regeneration. A tissue culture system for barley that appeared to be largely genotype independent seems to be the ovule culture technique (Holm et al. 1995), which however requires highly specialised resources and skills. A recent transcript-derived marker barley map based on ESTs was used to locate QTL for barley green plant regeneration and identify candidate genes, which include a ferredoxin-nitrate reductase and genes involved in hormone response and synthesis in cell division and the cell cycle (Tyagi et al. 2010). The identification of these genes should be the next step to manipulate regeneration ability in barley.

5.3.2 Development of DNA Delivery Techniques

The use of *Agrobacterium*-mediated gene transfer which got quickly established for numerous dicots in the 1980s was not that successful with cereals, since wounding of differentiated cereal tissues does not lead to the wound response-induced dedifferentiation in wound-adjacent cells (Potrykus 1990). Thus, numerous other methods for DNA transfer into the regenerative competent cells were developed for cereals which were also applied to barley. Amongst these methods, direct DNA transfer into protoplasts was easily achieved due to the absence of the cell wall; however, the first report on successful culture and selection of transgenic barley callus lines was published only in 1991 (Lazzeri et al. 1991), and it took another 4 years that fertile transgenic barley plants were generated via the protoplast approach using polyethylene glycol-mediated DNA uptake (Funatsuki et al. 1995; Kihara et al. 1998), followed by electroporation (Salmenkallio-Marttila et al. 1995) and microinjection in zygote protoplasts (Holm et al. 2000). Alternative methods were employed to circumvent difficulties of barley cell culture like imbibing of embryos in DNA (Töpfer et al. 1989), electrophoresis of DNA into germinating seeds (Ahokas 1989) and macroinjection of DNA into floral tillers or application of plasmid-DNA to stigmas (Mendel et al. 1990). However, no evidences for stable transformation were presented. Altogether, at the end of the 1980s, it emerged that those cells, which are transformable, are unable to regenerate and tissues like immature embryos with a high regenerative capacity lack methods to transform. A breakthrough was, however, the development of the particle gun (Klein et al. 1987), which enables direct transfer of DNA into regenerable tissues. The feasibility of microprojectile bombardment to transfer and express foreign DNA in barley cells was demonstrated by Mendel et al. (1989) and Kartha et al. (1989). Consequently,

the generation of fertile, transgenic barley was achieved using different targets like immature embryos (Wan and Lemaux 1994; Ritala et al. 1994; Hagio et al. 1995; Koprek et al. 1996; Jensen et al. 1996), microspores (Jähne et al. 1994; Leckband and Lörz 1998; Shim et al. 2009), embryogenic callus from immature embryos (Cho et al. 1998; Manoharan and Dahleen 2002) and mature embryos (Um et al. 2007) as well as in vitro shoot meristematic cultures derived from germinated seedlings (Zhang et al. 1999).

Successful transformation of cereals with *Agrobacterium* could be achieved due to the known advantages and also utilisation of hyper-virulent *Agrobacterium* strains as well as vectors containing extra copies of *vir* genes which together have helped in overcoming the restricted compatibility of the *Poaceae*. The progress made with rice (Hiei et al. 1994) and maize (Ishida et al. 1996) paved the way for barley also. Tingay et al. (1997) first demonstrated the suitability of *Agrobacterium tumefaciens*-mediated transformation for barley using immature embryos, and the method was optimised with regard to in vitro culture conditions (Trifonova et al. 2001; Bartlett et al. 2008) and factors influencing wounding and coculture (Shrawat et al. 2007). Additionally, the feasibility of other targets like embryogenic callus (Wang et al. 2001), ovules (Holme et al. 2006) and androgenetic pollen (Kumlehn et al. 2006) for *Agrobacterium*-mediated transformation was also explored resulting in a constantly growing number of reports in terms of stable expression of alien genes. A comparative analysis of transgenic barley plants generated via particle bombardment as well as via *Agrobacterium*-mediated DNA delivery clearly revealed a higher transformation efficiency, low-copy integration (between one and three copies in 100 % of the lines) and a stable inheritance of the T-DNA as a simple Mendelian trait for the *Agrobacterium*-derived lines (Travella et al. 2005). Experimental results of a large-scale study using Southern analysis indicated vector backbone integration in 48 % of the transgenic lines derived from *Agrobacterium*-mediated transformation of immature embryos in barley as described for other plants (Lange et al. 2006). Likewise, the twin T-DNA strategy based on transformation with an *A. tumefaciens* vector containing two adjacent T-DNAs thus enabling segregation of the selectable marker gene away from the gene of interest was also successfully applied for barley. The method represents a powerful approach for elimination of the selectable marker gene (Matthews et al. 2001).

In most cases barley transformation yielded in the regeneration and selection of heterozygous transgenic plants. Nevertheless, the doubled haploid (DH) lines are important tools for breeding and analysis of gene function. Basing on a huge knowledge in barley androgenesis, protocols were provided for inducing homozygosity in transgenic barley lines using microspore culture (Ritala et al. 2005). Importantly, it was demonstrated, that this is also a practicable and efficient approach for production of selectable marker-free, homozygous transgenic barley plants (Coronado et al. 2005).

Recently, a substantial increase in the transformation rate of barley was achieved due to a detailed study including comparison of *Agrobacterium* strains under diverse experimental conditions and the use of relatively high concentrations of L-cysteine and acetosyringone as supplements for cocultivation. This powerful protocol

enables a transformation efficiency up to 86.7 stable transgenics per 100 immature embryos inoculated with *A. tumefaciens* which was never described before (Hensel et al. 2008). Besides that, the integrated T-DNA copy numbers are typically low, the inheritance of the transgenes is according to the Mendelian rules, and the protocol is applicable for other genotypes and breeding lines also.

5.3.3 Targeted Expression of Alien Genes

The significant progress achieved in the last decade in barley transformation is correlated to elucidation of mechanisms that control transgene expression with respect to (1) strength, (2) cell and tissue specificity, (3) developmental specificity and (4) environmental effects. Thus, the choice of the promoter is of primary importance. Whereas, for development and optimisation of gene transfer methods in barley, the widely used constitutive promoters from the cauliflower mosaic virus gene (*35S*), rice actin 1 gene (*Act1*) and maize polyubiquitin gene (*ubi-1*) mainly were employed (Wan and Lemaux 1994; Jähne et al. 1994; Tingay et al. 1997), numerous specific promoters were isolated and introduced in cereals transiently and stably (Hensel et al. 2011). The first report for barley was a homologous approach to functionally validate the expression of barley high-pI α-amylase gene promoter and signal peptide-coding region fused to a hybrid bacterial thermostable (1,3-1,4)-β-glucanase. Nearly 75 % of grains harvested from primary transformants synthesised the gene of interest (Jensen et al. 1996). Likewise, the expression of a cloned fragment from the seed-specific β-amylase gene from barley was confirmed using the β-glucuronidase gene (*gus*) in a homologous system, and GUS activity was found in the subaleurone endosperm during seed maturation (Okada et al. 2000). Endosperm-specific expression during grain maturation in transgenic barley was detected analysing the barley B1 hordein (*Hor2-4*) and D-hordein (*Hor3-1*) promoters (Cho et al. 1999b, 2002; Furtado et al. 2009), a rice glutelin B1 (*GluB-1*) promoter (Patel et al. 2000) and two high-molecular-weight glutenin (*HMW-Glu*) promoters (Schünmann et al. 2002; Zhang et al. 2003; Furtado et al. 2009). Besides that, in a study with the green fluorescent protein gene (*gfp*) as a reporter, the wheat early methionine (Em) promoter was evaluated which maintained endosperm-specific expression in barley (Furtado and Henry 2005) suggesting its ability as a strong promoter to direct transgenes in specific tissues of barley. Furthermore, the oat globulin *AsGlo1* promoter region (960 bp) and a 251 bp fragment were used to produce transgenic barley. The mechanism of its specificity is different from that observed in glutelin and prolamin promoters due to a novel interrupted palindromic element (Vickers et al. 2006). The promoters of two rice genes (*OsPR602* and *OsPR9a*) fused to *gus* were also analysed in stably transformed barley, which displayed activity in early grain development with the strongest expression in endosperm transfer cells during the early stages of grain filling (Li et al. 2008). Likewise, the promoter of *ZmMRP-1*, a maize endosperm transfer cell-specific transcriptional activator, which plays a central role in the regulatory pathways controlling cell differentiation,

was introduced in barley. GUS activity was detected in the developing modified aleurone layer which indicates that the promoter responds to functional, transport-related signals (Barrero et al. 2009).

Targeted gene expression is a critical step to combat fungal pathogens in barley and any other crop. The promoter of the *Lem2* gene of barley, which encodes a lectin-like protein that is strongly upregulated by salicylic acid and is preferentially expressed in lemma, palea and coleoptile, was analysed. Promoter/*gfp* reporter constructs revealed cell- and development-specific expression of *gfp* in lemma/palea, glumes, coleoptile, auricle and ligule (Abebe et al. 2006). Another option in this regard is the promoter of a lipid transfer protein (*ltp6*) which has been cloned from barley. Different promoter deletion constructs were examined using *gfp,* and strong expressions in the ovaries and pericarp epidermis and during embryogenesis and germination were detected reflecting the expression pattern of the native gene therefore being suited for targeted disease resistance (Federico et al. 2005).

Recently, the promoter of the germin-like protein (*GER4*) was identified, which is involved in the pathogen-associated molecular pattern of barley leaf epidermis attacked by the powdery mildew fungus *Blumeria graminis* f. sp *hordei. GER4c* promoter responds with a high transcript dosage due to pathogen attack and seems to be a powerful tool to engineer disease resistance in cereals (Himmelbach et al. 2010).

Besides the availability of numerous specific promoters useful for controlling gene expression in cereals and establishment of high-throughput *Agrobacterium*-mediated transformation protocols, the demand for binary vectors has increased since these enable an easy insertion of promoters, effector sequences and selectable markers. For this purpose a set of modular binary vectors has been developed (Himmelbach et al. 2007).

5.4 Achievements in Transgene Technology in Barley

In the last decade tremendous progress has been made in genetic transformation of barley. There are numerous reports of both applied and basic nature, which imply that barley transformation is now optimised and a routine. Consequently, barley has emerged as a model plant of the *Triticeae* tribe (Saisho and Takeda 2011) and proves that elucidation of gene function is no longer restricted to the dicots.

5.4.1 Disease Resistance

Like other crops, barley is adversely affected by bacteria, fungi and viruses which cause a great variety of diseases. Depending on several factors like climatic conditions and crop protection measures adopted, losses due to pests are still high globally. In a study published in 2006, the global total potential loss for wheat varied from 26 to 29 %, whereas for maize and rice, it was 31–37 %, no figures were given

for barley (Oerke 2006). Nevertheless, a few data are available for individual countries and pests. Taking the example of barley yellow dwarf virus infection on yield and malting quality of barley in the USA, various aspects have been comprehensively examined and losses up to 40 % have been reported (Edwards et al. 2001). Economic losses for barley resulting from impacts of Fusarium head blight (FHB) were assumed to be up to 55 % for North Dakota and Minnesota from 1998 to 2000 (Nganje et al. 2001). Recently, in an assessment on the losses caused by diseases alone to the Australian barley industry, it was estimated that pathogens caused an average loss of 19.6 % of the average annual value of the barley crop in the decade 1998–1999 to 2007–2008 (Murray and Brennan 2010).

5.4.1.1 Fungus Resistance

A large part of research in barley genetic engineering is aimed at increasing fungal resistance. The first transgenic approach to increase fungal resistance in barley was transformation of the stilbene synthase gene of *Vitis vinifera*, resulting in the expression of the phytoalexin resveratrol capable of detoxifying fungal toxins. Pathological experiments indicated an enhanced resistance of T_1 plants in a detached leaf assay after inoculation with *Botrytis cinerea* (Leckband and Lörz 1998). In an effort to combat stem rust caused by *Puccinia graminis* f. sp. *tritici* in barley, the *Rpg1* gene for resistance to stem rust was introduced in a highly susceptible cultivar. A single copy of the gene conferred resistance against stem rust, and progenies from several transformants segregated in a 3:1 ratio for resistance/susceptibility, as expected. Therefore, it was demonstrated that the functional *Rpg1* gene isolated by map-based cloning coded for stem rust resistance (Horvath et al. 2003). On contrary, recently it was reported that transgenic barley lines overproducing functional RPG1 protein due to insertion of four or five copies responded with susceptibility to stem rust probably caused by the failure to degrade the RPG1 protein (Chai et al. 2012). Similarly, the maize *Rp1-D* gene, which confers race-specific resistance against *Puccinia sorghi* isolates containing a corresponding *avrRp1-D* avirulence gene, was inserted into barley but did not result in novel resistances when these plants were challenged with isolates of barley leaf rust *P. hordei* (Ayliffe et al. 2004).

The interaction of barley with the powdery mildew fungus *Blumeria graminis* f sp. *hordei* is presently the subject of intense research. Here, the polymorphic *Mla* locus harbouring race-specific resistance (R) genes is involved. To gain insights into *Mla*-mediated resistance, epitope-tagged Mla-variants, whose expression is driven by native regulatory sequences, were used for generation of transgenic barley lines. The findings show a reversible and salt concentration-dependent distribution of the intracellular MLA proteins in soluble and membrane-associated pools. The data demonstrate that *Rar1* encoding a intracellular Zn^{2+} binding protein positively controls steady levels of MLA resistance proteins and leads to accumulation of MLA6, thus enabling effective resistance (Bieri et al. 2004). Furthermore, stable over-expression of the constitutively activated barley RAC/ROP protein, RACB, reveals the enhancement of susceptibility to powdery mildew concluding that RACB might be involved in signalling in response to biotic stress (Schultheiss et al. 2005).

Besides that, the involvement of *HvRBOHF2*, a respiratory burst oxidase homologous NADPH oxidase gene, as well as *HvBI-1*, a BAX inhibitor with function in negative control of programmed cell death, was analysed for the interaction of barley and the powdery mildew fungus. Transgenic barley plants were developed with stably knocked down expression of *HvRBOHF2* which were unable to contain wound-induced cell death and revealed developmental alterations from the three-leaf stage onward. The results suggest that RBOHF2 is required for normal development of penetration resistance to the fungus (Proels et al. 2010). Moreover, barley plants carrying an *HvBI-1* RNA interference (RNAi) construct having lower levels of *HvBI-1* respond with less susceptibility to powdery mildew than wild-type plants accompanied by enhanced resistance to penetration by *B. graminis* f. sp. *hordei* at the cellular level (Eichmann et al. 2010). In contrast, transgenic barley plants over-expressing the cell death-regulating BAX inhibitor *HvBI-1* display suppression of defence response and resistance to *B. graminis* f sp. *hordei*; however, young seedlings were more resistant to *F. graminearum*. The authors concluded that the life cycle of the fungus influences the outcome of the effect of *HvBI-1* (Babaeizad et al. 2009).

Barley plants over-expressing the *HvBI-1* were a valuable tool to investigate the relationship between the fungus *Piriformospora indica* and barley. The endophytic fungus induces root resistance against head blight caused by *Fusarium culmorum* and also systemic resistance to powdery mildew via an unknown mechanism. Cytological and molecular evidences suggest that *P. indica* needs dead host cells for proliferation which progresses as the tissues mature. The expression level of the cell death regulator *HvBI-1* influences development of *P. indica* in barley. Fungal proliferation was remarkably reduced in the transgenic lines indicating that *P. indica* requires host cell death for proliferation (Deshmukh et al. 2006).

Another approach being explored is the use of pathogenesis-related (PR) proteins known to be associated with degradation of structural components of pathogenic filamentous fungi. Transgenic barley plants were generated by co-bombardment with two plasmids, one containing a rice (*Oryza sativa* L.) chitinase gene (*chi11*) and another carrying a rice thaumatin-like protein gene (*tlp*). From T_1 plants expressing both the proteins, T_3 homozygous lines were developed that co-express both antifungal proteins (Tobias et al. 2007). These lines when tested for many years exhibited reduced *Fusarium* head blight (FHB) incidence (Dahleen et al. 2011). The fungus also produces the mycotoxin deoxynivalenol (DON) which inhibits protein synthesis and is harmful to humans and animals and therefore reduces crop quality. A strategy to reduce DON accumulation in the grains focussed on introduction of *Tri101*, which encodes a 3-OH trichothecene acetyltransferase that converts DON to a less toxic acetylated form in barley. T_3 and T_4 progenies of three independent transgenic lines with *Tri101* showed a reduction in DON concentration (Manoharan et al. 2006). These lines were backcrosses and two of the resultant lines consistently showed a 40 % reduction in DON (Dahleen et al. 2011).

Another serious disease in barley is root rot caused by *Rhizoctonia solani* and *R. oryzae*. Transgenic barley liners have been developed to ubiquitously express a codon-optimised 42-kDa endochitinase *cThEn(GC)* from *Trichoderma harzianum* (Wu et al. 2006). Chitinases from this soil fungus effectively break down chitin, the main constituent of fungal cell walls of mature hyphae, conidia, chlamydospores

and sclerotia. The transgenic lines of barley displaying resistance to *Rhizoctonia* were analysed in field to monitor possible side effects of the genetic modification compared to the parental cultivar Golden Promise. Moreover, to assess influence of normal genotypic variation a second cultivar Baronesse was included in the study. Using parallel transcriptome and targeted metabolome profiling, as well as nontargeted metabolite fingerprinting, the data exhibited that cultivar-specific differences remarkably exceeded the effects caused by the transgene expression (Kogel et al. 2010).

A further interesting attempt to engineer disease resistance in barley against fungal plant pathogens is the use of antifungal peptides from insects. The suitability of metchnikowin, an antimicrobial peptide from *Drosophila melanogaster*, was evaluated for its resistance properties against damaging fungi. The transformed barley harbouring the metchnikowin gene showed increased resistance to powdery mildew, FHB and root rot. Additionally, accumulation of metchnikowin was also detected in plant apoplastic space specifying that the insect signal peptide is functional in monocotyledons (Rahnamaeian et al. 2009).

5.4.1.2 Virus Resistance

One of the most serious viral diseases of cereals worldwide is barley yellow dwarf (BYDV). Since sources of natural resistance to this virus are rare (Ordon et al. 2005; Kosová et al. 2008), the use of virus-derived transgenes was amongst the early approaches. Constructs containing the coat protein of several isolates of BYDV together with selectable markers were used resulting in some resistant barley plants (Wan and Lemaux 1994; McGrath et al. 1997); however, unfortunately resistance was not stable. Further experiments succeeded in transformation of barley with transgenes encoding an hpRNA derived from BYDV-PAV polymerase sequences, and one-third of the independently transformed lines exhibited very high resistance to BYDV-PAV (Wang et al. 2000). This was followed by the transfer of transgenes derived from BYDV and cereal yellow dwarf virus (CYDV) in an elite Australian barley cultivar. While there was considerable variability amongst the virus levels in different transgenic lines developed, some of the plants containing transgenes showed reduced virus symptoms (Wang et al. 2001).

The feasibility of using the barley "eukaryotic translation initiation factor 4E" (*Hv-eIF4E*), which was identified as a candidate for resistance gene function by physical mapping, was also analysed. It could be shown that *Hv-eIF4E* confers multiallelic recessive *Bymovirus* resistance in barley (Stein et al. 2005).

5.4.2 Abiotic Stresses

Significant yield losses are caused in barley by various abiotic stresses including drought, flooding, salinity, wind and temperature extremities and climate change in envisaged to increase the problem further (Gregory et al. 2009). Physiological,

biochemical and molecular approaches have been used to dissect the response of plants to abiotic stresses describing effectors, regulatory genes and gene networks emphasising the pivotal role of transcription factors (Nakashima et al. 2009).

Approximately 40 % of agriculturally used area is covered by acid soils strongly limiting productivity. Due to acidity, aluminium is solubilised which rapidly inhibits root growth and thereafter water as well as nutrient uptake. A malate transporter from wheat (*ALMT1*) enabling malate efflux was introduced in barley and the transgenic lines when evaluation for response to aluminium (Al) stress demonstrated a high level of aluminium tolerance in hydroponic culture as well as on acid soils (Delhaize et al. 2004). Further analysis on relation between aluminium resistance and phosphorus nutrition in wild-type and transgenic plants expressing *TaALMT1* revealed a higher efficiency of the transgenics in taking up phosphorus on acid soil. In addition, a higher root growth, shoot biomass and grain yield were observed for the *TaALMT1*-plants in comparison to the control when grown up to maturity on the same soil (Delhaize et al. 2009). In another study, Li et al. (2010) found increased aluminium resistance in roots of transgenic barley over-expressing *Phalaris coerulescens* thioredoxin gene (*PTrx*) (Li et al. 2010).

Um et al. 2007 generated transgenic barley plants containing cDNA from *Arabidopsis* nucleoside diphosphate kinase 2 (*AtNDPK2*). They observed 10 % reduction in membrane damage in the transgenic plants caused by methyl viologen which indicated the expression of *AtNDPK2*. Similarly, an alfalfa aldo-keto reductase (*MsALR*) which can detoxify lipid peroxide degradation products was over-expressed in barley to eliminate toxic reactive aldehyde products from cells after oxidative stress. The cellular stress response of the transgenic plants was investigated in transient assay estimating damaged cells microscopically using fluorochromes and determining chlorophyll as well as carotenoid content (Nagy et al. 2011). In all cases transgenic plants outperformed controls after applying stress.

Recently, two dehydration-responsive proteins (DREBs) from wheat were analysed for their potential to modify transcriptional regulation of drought and cold stress in barley (Morran et al. 2011). Constitutive over-expression of *TaDREB2* and *TaDREB3* resulted in stable transformed plants which responded significantly better to drought and cold stresses compared to the controls. However, these showed negative impacts on developmental parameters like stunted growth, dwarfism, delayed flowering and smaller spikes. In contrast, it was observed that the drought-stress-inducible *ZmRab17* promoter is quickly and strongly activated by drought causing little or no adverse developmental traits (Morran et al. 2011). Besides that, the feasibility of the transcription factor *Osmyb4* from rice characterised as a central point of a large transcriptional network was also evaluated for modulation of stress response in barley. Progeny of transgenic lines harbouring *Osmyb4* under control of the *Arabidopsis* cold-inducible promoter *cor15a* was exposed to freezing, and the damage was determined through analysis of chlorophyll fluorescence parameters. During germination pronounced differences were observed concerning higher vigour to hypoxia combined with cold stress compared with the controls. These data support an involvement of *Osmyb4* in flooding tolerance and in alleviation of germination under adverse environmental conditions (Soltész et al. 2012).

5.4.3 Improvement of Product Quality and Plant Productivity

5.4.3.1 Polysaccharides

A large part of applied research is focussed on alteration of processing quality of barley grains since the starchy endosperm contributes to about 80 % of the total grain weight. The linear polysaccharides (1,3-1,4)-β-glucans are the major constituent of endosperm cell walls in barley. Thus, enzymatic mobilisation of endosperm storage constituents requires degradation of these cell walls for a high and efficient use of barley grains for feed and malting.

In early attempts barley was manipulated to express a codon-optimised bacterial thermostable (1,3-1,4)-β-glucanase to improve the digestibility of the grains in monogastric animals like poultry. About 75 % of grains from primary transformants synthesised thermostable (1,3-1,4)-β-glucanase and inheritance of transgene expression was reported in scutellum and aleurone of germinating seeds (Jensen et al. 1996, 1998; Horvath et al. 2000; Xue et al. 2003). Likewise, a fungal xylanase gene under the control of an endosperm-specific promoter from cereal storage protein was introduced in barley to produce plant cell wall polysaccharide-hydrolysing feed enzymes in the endosperm to replace the later addition of microbial produced xylanases to the feed thus reducing the costs (Patel et al. 2000).

An important aspect regarding malting quality in barley is increasing thermostability of enzymes, since in malting and brewing industries, the grains are exposed to temperatures above 70 °C. In this direction, a mutant thermostable β-amylase gene generated by site-directed mutagenesis was used to design transgenic barley plants. An increase in thermostability by 11.6 °C compared to the original enzyme was obtained which was stably transmitted to progeny (Kihara et al. 2000). Similarly, a gene encoding for a thermotolerant fungal endo-(1,4)-β-glucanase (Nuutila et al. 2002) and the heat-stable alkalophilic *Bacillus* α-amylase (Tull et al. 2003) was also introduced in barley resulting in an enhancement of α-amylase activity by 30–100 % compared with the control.

In another approach the wheat thioredoxin *h* gene (*wtrxh*) driven by a seed-specific promoter was over-expressed in barley to gain insight in its putative role in germination and seedling development. The results demonstrated an increased activity of a starch-branching enzyme of the endosperm, pullulanase specifically hydrolysing α-1,6-linkages in starch (amylopectin) during germination and seed development (Cho et al. 1999a). In addition, these plants displayed enhanced root and shoot growth in the presence of 2 mM sodium selenite suggesting that overexpression of thioredoxin *h* could be a tool for application in the remediation of polluted soils (Kim et al. 2003).

Further aspects of interaction between starch metabolism and plant development were studied by antisense downregulation of the barley limit dextrinase inhibitor (LDI). Transgenic barley plants were developed to investigate the function of LDI. In homozygous antisense lines, an increased LD activity was observed in developing and germinating seeds accompanied by unpredicted pleiotropic effects on

numerous enzyme activities, reduced numbers of the small B-type starch granules and reduced amylose relative to amylopectin levels (Stahl et al. 2004). Another transgenic approach using RNAi-mediated silencing was addressed to the starch-branching enzymes, SBE IIa and SBE IIb, to define structure of amylose and amylopectin in the barley endosperm. The data suggested that a reduction in the expression of both SBEs was necessary to significantly increase amylose content in comparison to the wild types (Regina et al. 2010). Very recently, the simultaneous suppression of all starch-branching enzyme genes (*SBE I*, *SBE IIa* and *SBE IIb*) using a chimeric RNAi hairpin was described. Carciofi et al. (2012) succeeded in generation of barley with amylose-only starch granules which were irregularly shaped. The grains of the transgenic lines germinated like the controls displayed comparable high yield, but growth was delayed suggesting an important physiological role of amylopectin (Carciofi et al. 2012).

Of late, the (1,3-1,4)-β-D-glucan, a major constituent of the cell wall of cereals and grasses, has gained renewed interest of plant scientists due to its beneficial effects on human health and as a valuable source of fermentable sugars for bioethanol production (Newton et al. 2011). Investigations were concentrated on increasing the (1,3-1,4)-β-D-glucan levels by over-expression of barley cellulose synthase-like family (*CslF*) cDNAs under control of an oat globulin promoter or a constitutive promoter. An enhanced amount of 80 % (1,3-1,4)-β-D-glucan in grains of transgenic barley was obtained in case of the endosperm-specific promoter, whereas gene expression driven by the constitutive promoter resulted in sixfold higher levels of (1,3-1,4)-β-D-glucan in vegetative organs and similar levels in grains compared with the control (Burton et al. 2011). A further analysis was conducted to explore the role of α-glucosidase in germinating barley grains. In seedlings harbouring an RNA interference silencing cassette for *HvAgl97*, α-glucosidase was lowered up to 50 %. The findings indicate that the α-glucosidase *HvAGL97* is the major endosperm enzyme catalysing the conversion of maltose to glucose but is not required for starch degradation in contrast to results from biochemical assays with glucosidase inhibitors (Stanley et al. 2011).

5.4.3.2 Proteins

With respect to manipulation of seed storage proteins, a cDNA encoding the γ-zein protein of maize driven by an endosperm cell-specific promoter was used to determine deposition pattern and impact on grain properties. In transgenic barley, an accumulation of nearly 2 % of γ-zein of the total grain nitrogen was reported corresponding to 4 % of the total protein fraction. However, no effects on grain texture like hardness or vitreousness were described (Zhang et al. 2003). A very interesting approach is the production of a therapeutic protein in barley grains. Since barley is a major constituent of feed for domestic animals, its suitability as a source for oral vaccination against porcine diarrhoea caused by F4-positive enterotoxigenic *Escherichia coli* (ETEC) strains was explored. A protective immune response against the disease is inducible by F4 fimbriae or FaeG. Transgenic barley was

designed expressing the F4 fimbrial adhesin FaeG in a glycosylated form in the endosperm up to 1 % of total soluble protein. The recombinant protein was resistant to storage and simulated digestive conditions. In addition, glycosylation did not negatively influence immunogenicity since erFaeG was able to induce F4 fimbria-specific antibodies in mice (Joensuu et al. 2006).

The alteration of the amino acid composition of barley is important to improve the feeding quality of its grains and also to avoid the use of large-scale protein supplements derived from soybean or microbes. Efforts were undertaken to increase the content of essential amino acids, especially lysine, threonine and methionine in barley. Lange et al. (2007) aimed at selective suppression of C-hordein synthesis, the storage protein with the lowest nutritional value by an antisense approach. From the 35 primary transformants, five lines were selected for comprehensive analysis using SDS-PAGE and reverse phase HPLC. Their data demonstrated a relative reduction in the content of C-hordeins combined with a relative rise in the synthesis of other storage proteins in the mature grain. An increase was found in lysine, threonine and methionine content (16, 13 and 11 %) indicating antisense-mediated suppression of C-hordein synthesis as a promising approach (Lange et al. 2007). The data were confirmed by a transcriptomic analysis of one of the antisense C-hordein lines using a grain-specific cDNA microarray (Hansen et al. 2007). More recently, another strategy to manipulate lysine content in barley grains has also been successfully applied. The key enzyme involved in the regulatory step for lysine biosynthesis dihydrodipicolinate synthase (*dapA*) from *E. coli* was employed. Analysis revealed T_1 lines with enhanced level of lysine in leaves as well as T_2 lines with higher amount in seeds relatively to the wild type (Ohnoutkova et al. 2012).

5.4.3.3 Micronutrients

Research activities have been increasingly focussed in barley on combating micronutrient deficiency with regard to the plant as well as to the subsequent consumer using this plant product. In an attempt to increase phosphate uptake in barley plants, a high-affinity phosphate transporter was over-expressed in barley, but this did not enhance phosphate uptake in transgenic plants (Rae et al. 2004). In contrast, over-expression of an *Arabidopsis* zinc transporter (*AtZIP1*) resulted in a rise of short-term zinc uptake after zinc deficiency and seed zinc content thereby improving its nutritional quality (Ramesh et al. 2004). Very recently, the genetic modification of barley for improvement of phytase activity was reported applying the cisgenesis concept. Phytases are essential enzymes for the sequential release of phosphate groups from phytic acid thereby providing bioavailable phosphate which otherwise could not be used from monogastric animals. Enhanced phytase activity up to 2.6- to 2.8-fold was found in the seeds from lines homozygous for the insert. Besides that, the activity levels were stable over the three generations assayed (Holme et al. 2012).

5.4.3.4 Plant Productivity

For acceleration of plant development, the *vhb* gene encoding *Vitreoscilla* haemoglobin (VHb) known to improve cellular respiration and efficient energy generation during oxygen-limited growth was inserted in barley. Nevertheless, constitutive *vhb*-expressing plants failed to fulfil the expectations (Wilhelmson et al. 2007). In an attempt to develop early flowering barley plants, the natural early flowering time allele Cape Verde (Cvi) of Cryptochrome2 (*AtCRY2-Cvi*) gene from an *Arabidopsis* was employed. Seeds from T_1 plants were evaluated which recorded more than 25 days earlier flowering and day-length insensitivity as compared to the controls (El-Din et al. 2011).

Zalewski et al. (2010) concentrated on silencing the expression of cytokinin oxidase/dehydrogenase (*HvCKX1*) applying RNAi-based technology to elucidate the function of the gene in barley. The authors succeeded in generating more than 50 lines from which nearly 80 % displayed significantly reduced CKX activity in bulked samples of their T_1 roots. A positive relationship between enzyme activity and plant productivity was determined, reflected as the yield, the number of seeds per plant and 1,000 grain weight. Consequently, decreased CKX activity led to a higher plant yield and root weight (Zalewski et al. 2010). Furthermore, silencing of *HvCKX2* resulted in different phenotypes depending on the transformation method. *Agrobacterium*-mediated gene transfer yielded silenced lines with higher productivity, whereas biolistic silenced lines exhibited low productivity and disturbances in plant development (Zalewski et al. 2012).

5.4.4 Barley Grains as a Bioreactor

Cereal grains offer an excellent opportunity for production of recombinant pharmaceutical proteins since they were bred to accumulate and store large amounts of carbohydrates and proteins. Molecular breeding in cereal crops with respect to expression level, protein authenticity, downstream processing and purification as well as regulatory issues has been comprehensively covered previously (Ramessar et al. 2008; Hensel et al. 2011). Barley is of great importance because of it being a self-pollinator, unable to generate fertile hybrids with related species and wide adaptation with an extremely wide geographic distribution.

The applicability of barley to produce heterologous proteins was initially demonstrated using a protein-engineered thermostable (1,3-1,4)-β-glucanase and a fungal xylanase (Nuutila et al. 1999; Horvath et al. 2000; Patel et al. 2000; Xue et al. 2003). Consequently, this potential was explored for substances, for which traditional production methods are expensive, inefficient and laborious. Thus, an antibody-fusion protein used to detect HIV-1 in human blood by causing rapid agglutination was expressed in barley. Schünmann et al. (2002) succeeded in high-level expression of an antiglycophorin single-chain antibody fused to an epitope of the HIV virus in seeds of barley, which can substitute the SimpliRED™ diagnostic reagent. Additionally, the yield in barley (150 µg/g of regent per gramm) exceeded

amounts expressed in transgenic tobacco leaves and potato tubers which were evaluated in parallel (Schünmann et al. 2002). Besides that, transgenic barley plants comprising genes for production of human antithrombin III, α1-antitrypsin, lysozyme, serum albumin and lactoferrin were reported (Stahl et al. 2002). Transgenic barley plants expressing human lactoferrin (*hLF*) were also described from other groups. Western blot analysis of leaf tissue from T_0 plants documented the expression of the recombinant human lactoferrin (Kamenarova et al. 2007), whereas Tanasienko et al. (2011) provided proof for the presence of the gene *hLF* fragment in leaves of T_0 plants by PCR.

Eskelin et al. (2009) and Erlendsson et al. (2010) presented further convincing evidences that transgenic barley seeds can be utilised as a bioreactor. The successful expression of both the recombinant full-length and the 45-kDa fragment of human collagen-type I α-1 chain (*rCla1*) in barley seeds was obtained screening three promoters. The proteins were further targeted to the endoplasmic reticulum to enhance the expression levels of recombinant proteins as previously shown (Horvath et al. 2000). The glutelin promoter was superior in yielding 45 mg recombinant protein per kg dry seeds in the best lines compared to 15 mg/kg caused by the ubiquitin promoter (Eskelin et al. 2009). Moreover, the Orfeus™ expression system developed by ORF Genetics (Reykjavik, Iceland) was used to produce recombinant human Flt3 ligand in barley grains. For that purpose, the cDNA of human Flt3 ligand, a growth factor necessary for proliferation and differentiation of stem cells, with an HQ_6-tag under control of the hordein promoter was used. High expression of biologically active Flt3 ligand with a yield comparable to prokaryotic production was reported (Erlendsson et al. 2010).

Consequently, this huge potential relying on plant-based recombinant protein production, in general and in cereal-based production in particular, is exploited commercially by several companies like Maltagen Forschung GmbH (Andernach, Germany), ORF Genetics (Reykjavik, Iceland) and Ventria Bioscience (Fort Collins, USA) and is reflected in patents (Stahl et al. 2009). Thus, the barley endosperm is an efficient bioreactor for pharmaceutical proteins like cytokines, oral vaccines, growth factors and food additives.

5.4.5 Elucidation of Gene Function

The enormous progress made in barley transformation in the last decade together with the dramatic advances in genome research due to new technologies as well as an ever-increasing number of data accessible in public databases (Sreenivasulu et al. 2008) has enabled the functional characterisation of candidate genes identified in functional genomic studies. Comprehensive determination of gene function via over-expression or reduction of gene expression up to the knockout of plant genes is no longer restricted to the model dicot *Arabidopsis thaliana*. Thus, insights in metabolic and regulatory networks possibly linked to agronomically important traits become important. Table 5.1 summarises examples for the elucidation of gene function made in barley so far.

Table 5.1 Elucidation of gene function in barley

Promoter specificity/ coding sequence	Effect	Reference
Wheat HMWGLU-1 D1 antisense *SnRK1* protein kinase	Promoter activity in seeds and anthers 50 % pollen: arrested at binucleate stage, contains little or no starch, and is non-functional, male sterility	Zhang et al. (2001)
HvGAMYB (transcription factor in barley aleurone and anthers)	Active in early anther development, over-expression led to decrease in anther size, male sterility	Murray et al. (2003)
jekyll (expressed in barley grain nucellar projection tissue)	RNAi: decelerates autolysis and cell differentiation within nurse tissues, no function of nuclear projection as main transport route for assimilates, irregular and small-sized seeds	Radchuk et al. (2006)
LOX2:Hv:1 with and without the chloroplast targeting signal	Over-expression: higher levels of jasmonic acid for lines with elevated levels of LOX-100 in chloroplasts and in cytoplasma, respectively	Sharma et al. (2006)
TaMSH7 mismatch repair gene	RNAi: results in reduced seed set	Lloyd et al. (2007)
Short Vegetative Phase (SVP)-like MADS-box genes	Ectopic expression: inhibition of spike development, floral reversion, florets at the base of the spike, delay of head emergence, inhibition of floral meristem identity	Trevaskis et al. (2007)
PpENA1 (sodium-pumping ATPase from the bryophyte *Physcomitrella patens*)	Over-expression: marked increase in levels of free amino acids, organic acids, and salicylic acid and in some sugars and fatty acids	Jacobs et al. (2007)
HvABA8′OH1 (ABA catabolism)	RNAi: reduced expression results in higher levels of ABA and increased dormancy	Gubler et al. (2008)
Whirly1 (encodes a nucleic acid-binding protein)	RNAi: plastid located Whirly1 functions primarily in RNA metabolism rather than as a DNA-binding protein	Melonek et al. (2010)

5.5 Problems and Prospects

5.5.1 Genotype Dependency

Despite significant progress achieved in the last decade in transfer of alien genes into *H. vulgare* using biotechnological tools, the strong genotype dependency is still a key problem and still hampers routine application of gene transfer to improve traits in a desired cultivar. A highly efficient and reproducible regeneration system using immature embryos is only available for the spring variety, Golden Promise, identified in the middle of the 1980s (Lührs and Lörz 1987). The responsiveness

of this genotype to regeneration protocols is convincingly demonstrated in a summary on barley transformation up to 2007 where nearly two-thirds of all reports used only this genotype (Schulze 2007). Dahleen and Manoharan (2007) pointed out that this model genotype is suitable for improvement of transgene technology but is not suited for evaluation of transferred genes in local field conditions owing to less agronomic value. In fact, from a practical point of view, agronomically superior cultivars adapted to the region should only be used despite their recalcitrance to tissue culture methods in order to achieve gains in terms of production and quality. A few successful reports are available for commercially important barley varieties including the Finnish elite cultivar Kymppi (Ritala et al. 1994; Nuutila et al. 1999); several German spring cultivars (Koprek et al. 1996; Sharma et al. 2006); the North American cultivars, Harrington, Galena and Conlon (Cho et al. 1998; Zhang et al. 1999; Manoharan and Dahleen 2002; Manoharan et al. 2006; Tobias et al. 2007); the elite Australian barley cultivars, Schooner, Chebec and Sloop (Wang et al. 2001; Murray et al. 2004); and a Bulgarian winter barley (Kamenarova et al. 2007). However, in all these studies transformation efficiency was reported to be low. Similarly, the transformation frequency in other spring and winter genotypes that were generated was also low despite considerable improvements of protocols for *Agrobacterium*-based transformation (Hensel et al. 2008). Recently, stable transformation of commercially important varieties from Saudi Arabia (El-Din et al. 2011), from the Ukraine (Tanasienko et al. 2011) and from India (Yadav et al. 2013) was reported reflecting the need for improvements of cultivars, which can be used effectively in areas with special conditions as described for instance for the Ukraine having also marshy woodlands, forest-steppe, and steppe (Tanasienko et al. 2011).

Holme et al. (2008) reported a genotype-independent method of DNA delivery using young barley embryos derived from in vitro cultured ovules as targets for *Agrobacterium*-mediated transformation. Nonetheless, this method is not suited for high-throughput and cost-oriented transformation technology due to its sophisticated and labourious protocol. Other possible targets are in vitro shoot meristematic cultures derived from germinated seedlings which can be induced with low genotype dependency (Ganeshan et al. 2003; Sharma et al. 2004). The feasibility of these meristematic cultures characterised by proliferation of tightly packed clusters of continuously multiplying axillary and adventitious buds (Fig. 5.1) was validated for barley also (Zhang et al. 1999).

5.5.2 Transgene Insertion and Stability

Another problem in barley transformation is transgene stability. Direct DNA delivery frequently results in multicopy integration, and rearrangement of the transgene and gene silencing often has been linked to this phenomenon (Vaucheret et al. 1998; Cho et al. 1999b, 2002; Bregitzer and Tonks 2003). Convincing evidences were provided by Travella et al. (2005) comparing transgenic barley lines generated by biolistics and *Agrobacterium*-mediated method. Sixty per cent of the particle

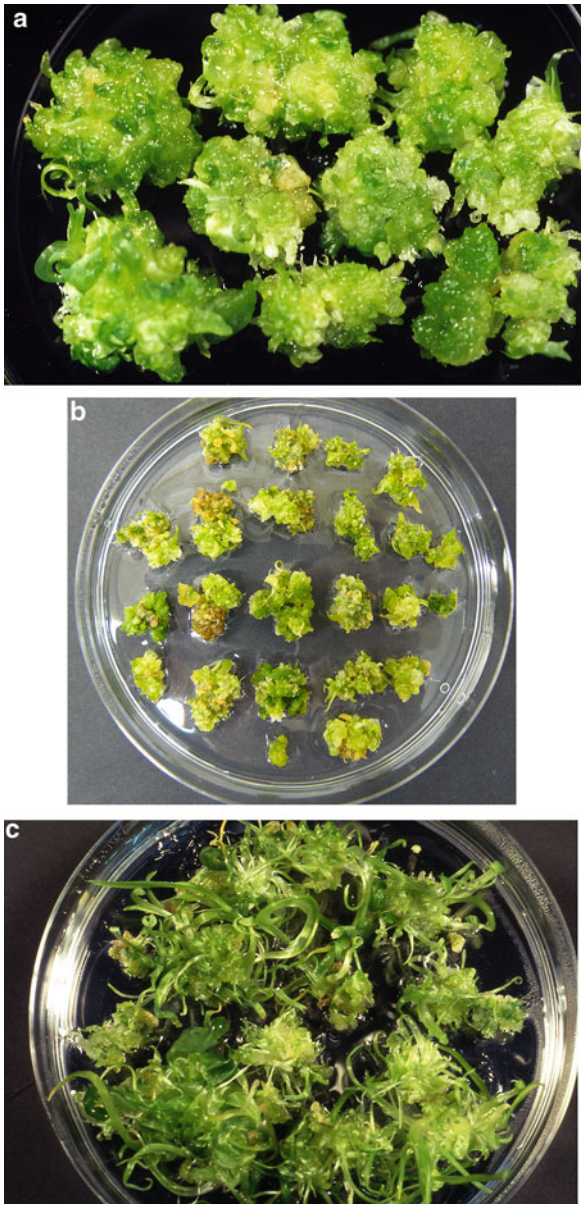

Fig. 5.1 Morphogenic response of cultures established from meristematic shoot segments from barley (*H. vulgare* cv. Lomerit) as described by Sharma et al. (2004). (**a**) Tightly packed clusters of continuously multiplying axillary and adventitious buds. (**b**) Green clumps of shoot buds on maintenance and proliferation medium 4 weeks after subculture. (**c**) Multiple shoot formation from bud clumps 2 weeks after transfer on regeneration medium

bombardment-derived lines integrated more than eight copies of the transgenes. Besides that, in all those lines extensive DNA rearrangements with multiple integrations were observed. In contrast, integration of only 1–3 copies of the transgenes with minimal rearrangements was detected in the lines produced by *Agrobacterium*-mediated method. Further, in case of *Agrobacterium*-based lines, analysis of

progeny revealed that the integrated T-DNA was inherited as a simple Mendelian trait and no silencing of the *bar* gene was observed in T_1 plants (Travella et al. 2005). The advantages of *Agrobacterium*-mediated gene transfer with respect to lower copy number for barley were also substantiated by Bartlett et al. (2008) and Hensel et al. (2008). The other possible reasons for silencing of transgenes could be promoter interference (Tobias et al. 2007) and methylation of the first untranslated exon and 5′ end of the intron in the *Ubi1* promoter complex (Meng et al. 2003).

A comprehensive study on transgene integration using fluorescence in situ hybridisation (FISH) from 19 independent barley lines revealed that transgene integration sites were found only on five of the seven barley chromosomes (Salvo-Garrido et al. 2004). Further, specific regions of the chromosomes 4H and 5H were detected containing clusters of transgene insertions, thus indicating a non-random pattern of integration. The data suggested that transgene insertions were preferentially located in gene-rich areas of the genome. A promising new tool to target a transgene to a specific locus in crops was recently presented by Shukla et al. (2009) using designed zinc-finger nucleases (ZFNs) that induce a double-stranded break at their target locus. The concomitant expression of ZFNs and delivery of a simple heterologous donor molecule resulted in precise targeted addition of a herbicide-tolerance gene at the intended locus in *Zea mays*, and genetic changes were transmitted to the progeny (Shukla et al. 2009).

Analysis of long-term stability of *gus* and *sgfp*(S65T) driven by the B1- and D-hordein promoter up to the T_9 generation revealed transgene stability in 93 % of the transgenic lines examined, while expression of *bar* under control of the maize ubiquitin promoter was found in only 60 % lines (Choi et al. 2003). Advanced generation of these lines containing the transgenes were crossed to obtain plants expressing multiple transgenes. Thus, a homozygous T_8 plant containing *gus* driven by the barley endosperm-specific B1-hordein promoter was crossed with another homozygous T_4 plant, carrying *sgfp(S65T)* driven by the barley endosperm-specific D-hordein promoter. PCR was used to monitor F_1 progeny for the transgenes *gus* and *sgfp*(S65T). Furthermore, functional expression of both transgenes was evaluated up to the F_4 generation. Localisation of transgenes by FISH revealed the same location of transgenes as in the parental plants (Choi et al. 2009).

5.5.3 Marker Gene Elimination

A requirement for the commercial use of genetically modified barley is the elimination of the selectable marker gene. For this purpose, several approaches have been evaluated. Matthews et al. (2001) investigated the twin T-DNA principle as already mentioned. An *Agrobacterium* vector comprising two contiguous T-DNAs, one with the gene of interest and the other one with the selectable marker gene, can be used to generate T_1 lines which have inserted the gene of interest and is free of the selectable gene (Matthews et al. 2001; Xue et al. 2003). This methodology is routinely used now. Another strategy aims at androgenetic generation (haploid technology)

of a segregating population of homozygous plants raised from pollen of primary transgenic barley plants produced via *Agrobacterium* infection of immature embryos. The results demonstrated that selectable marker-free homozygous transgenic plants can be efficiently generated (Coronado et al. 2005; Kapusi et al. 2013). However, even though haploid technology represents an elegant solution and accelerates time and resource efficiency of generating true-breeding, selectable marker-free transgenic barley, the major limitation is its strong genotype dependency. In both studies mentioned above, the variety Golden Promise was used which shows a poor response in pollen culture. Hence, culture conditions for immature pollen were elaborated. No promising reports on barley varieties are available which show a reasonable regenerative response of immature embryos and pollen. Therefore, the regeneration process has to be standardised for each and every genotype independently, which is a time-consuming process.

5.5.4 Field Trials and Risk Assessment

Assessment of genetically modified plants under natural field conditions is essentially required (1) to analyse transgene stability under natural environment, (2) to verify possible negative side effects of the introduced gene, (3) to monitor influence of natural genotypic variation, (4) to study ecological impacts, and (5) to evaluate the impact of environment on the expression of transgene. To analyse agronomic performance of genetically modified barley, the initial field trials were conducted in 1994 using T_2 generation of transformed plants. Compared to seed-derived Golden Promise plants, the transgenics were shorter and showed lower yield, smaller seed and a high variability amongst the individual plants (Bregitzer et al. 1998b). In another small-scale field experiment monitoring several agronomic traits, no differences were reported in the transgenic barley lines containing the *bar* gene and non-transformed control plants (Harwood et al. 1999). Yet in another study, reduced 1,000-grain weight and variable yield reductions were reported in transgene lines as compared to the Golden Promise cultivar (Horvath et al. 2001).

Field assessment also helps to adjudge the behaviour of a transgenic plant when it is taken to the natural field conditions. For instance, for the modification of the mycotoxin deoxynivalenol (DON) produced by the fungus *F. graminearum*, the transgenic T_3 and T_4 barley lines revealed a reduction of DON concentration in greenhouse test. However, this observation was not confirmed under field conditions, and possibly variations in temperature and humidity, inoculum and disease pressure could have overwhelmed the effects of *Tri101* against DON (Manoharan et al. 2006). Kogel et al. (2010) observed that cultivar-specific differences markedly exceed effects caused by the transgene expression as discussed above.

To study the level of gene flow, field trials were conducted with genetically modified homozygous barley lines harbouring the gene for neomycin phosphotransferase II in 1996 and 1997 in Finland. In these studies, while male sterile barley lines were used as recipients for pollen from the transgenics, normal male fertile

barley was also included to monitor transgene flow in normal barley. The results clearly indicated that the chance of cross-pollination to normal fertile barley varied from 0 to 7 % at 1 m distance, depending on weather conditions. However, the rate of cross-pollination declined rapidly with an increase in isolation distance; thus, in a range of 50–100 m distance, only a few seeds developed on male sterile barley due to cross-pollination from transgenic lines (Ritala et al. 2002; Nuutila et al. 2002). Studies on gene flow were also conducted under field conditions between transgenic and non-transgenic barley cv. Golden Promise in south-eastern Australia. The results indicated that outcrossing occurred at a rate of 0.005 % over a distance of less than 12 m, and therefore, the risk of gene flow between transgenic and non-transgenic barley at the field scale would be very low providing that crops were separated by a few metres (Gatford et al. 2006).

The tremendous progress made in barley genetic engineering is also reflected in the number of proposals for field trials. For the USA, 83 applications for release of transgenic barley in the period from 1993 up to 2010 were submitted. In contrast, there were only nine proposals for the European Union between 1996 and 2009 (GMO compass 2013). In all these cases the traits that have been targeted are fungal resistance, modified product characteristics and herbicide tolerance.

In Australia, currently a comprehensive research programme led by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) is underway which started in 2009 with approval of the Office of the Gene Technology Regulator (OGTR) to assess genetically engineered barley in small field plots in a 3-year test. Meanwhile six additional applications were approved (DIR094, DIR099, DIR102, DIR111, DIR112, DIR117) for release of GM barley lines containing genes for alteration of starch metabolism, for enhancement of the content of resistant starch and for improvement of nitrogen use efficiency and abiotic stress tolerance as approved by OGTR (http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/map; verified February 2013). In contrast, the number of field trials with genetically modified plants in general is declining in Europe (http://www.gmo-safety.eu/news/1416.plant-research-europe-genetic-engineering-field-trials.html; verified February 2013). A small field trial with genetically engineered barley producing the enzyme phytase is being carried out between 2011 and 2015 in the Czech Republic (Notification Number B/CZ/11/2) to verify stability of the transgene *phyA* in progeny (GMO Register 2013). Similarly, field trials with transgenic barley have also been conducted very recently (between 2010 and 2013) in Denmark and Sweden (GMO Register 2013). In the USA, 11 field tests were conducted between 2011 and 2012 focussing on *Fusarium* head blight resistance, *Rhizoctonia* resistance and increased nitrogen utilisation efficiency (details available on http://www.nbiap.vt.edu/).

5.5.5 Public Acceptance

One of the major hurdles in large-scale adoption of transgenic barley is its public acceptance. Since barley is mainly used for brewing and malting industry as well as

feed for animals, the inclusion of selectable markers like antibiotic or herbicide resistance is a cause of concern and invokes debates worldwide. Marker-free transgenic plants are one of the solutions to this issue (Matthews et al. 2001; Xue et al. 2003). Further, the gene flow between the transgenic barley and its wild counterpart also remains a concern despite careful investigations on this issue (Gatford et al. 2006). Additionally, apprehensions with regard to possible health risks by consumption of end products from genetically modified barley also exist thereby forcing the industry to take a wait-and-see stance. All these concerns have sometimes resulted in acts of vandalism, for example, in Europe (Kuntz 2012) and some of the field trials for barley have also been the targets of destruction (http://www.gmo-safety.eu/news/505.destruction-barley-trial-field.html; verified February 2013).

To circumvent the problems associated with the use of artificial gene combinations, the cisgenesis concept was developed (Schouten et al. 2006). The concept implies that the genetic material introduced in a plant should originate from the plant itself or from a species being crossable with that plant; thus, the gene pool for cisgenesis is the same as for classical breeding. Additionally, sequences from selectable marker genes or vector backbone sequences have to be absent. The feasibility of the cisgenesis concept was analysed for barley also aiming at improved phosphate bioavailability in the grains which are used as feed for monogastric animals such as pigs and chickens. A barley phytase gene (*HvPAPhy_a*) was used which is expressed during grain filling, and marker-free plant lines were recovered applying the marker gene elimination method. The insertion of the genomic clone for *HvPAPhy_a* resulted in lines with enhanced activity of phytase as discussed above (Holme et al. 2012), and field trials were conducted in 2012 (GMO Register 2013, Notification Number B/DK/12/01).

5.6 Conclusions and Future Prospects

Tremendous progress has been made in alien gene transfer to barley during the last two decades, converting it from an otherwise recalcitrant crop to a model for the *Triticeae*. Advances in distant hybridisation were achieved due to development of genomic tools and molecular techniques enabling marker-assisted selection and targeted backcrossing. The exploitation of genetic diversity through extensive screening programmes evaluating related wild species and landraces for valuable agronomic traits and sources of resistance to biotic and abiotic stresses provided an important tool for the generation of lines containing introgressed segments from wild species such as *Rym14(Hb)*, *Rym16(Hb)* and *Ryd4(Hb)* derived from *H. bulbosum* and conferring resistance to BaMMV, BaYMV and BYDV in barley.

Conventional breeding, of late, has been complemented by the biotechnological approaches of alien gene transfer, and these were particularly benefited from the establishment of *Agrobacterium*-mediated genetic transformation technology in combination with significant progress in genomic research. Consequently, functional characterisation of candidate genes for targeted manipulation of specific characters

is now possible and is extensively used for fundamental and applied research in barley. Nevertheless, despite development of numerous genetically modified barley lines with improvements in product quality, composition and resistance to stresses, most of these lines are still in the laboratory or in experimental field trials. If outcomes of the analyses made in Australian field trials are positive, commercial varieties will be available in around 2020 (http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/map; verified February 2013). Similarly, encouraging reports are also there from the trials being conducted in the USA. Therefore, it can be concluded that the recent developments made in distant hybridisation, standardisation of tissue culture protocols, establishment of *Agrobacterium*-mediated and other genetic transformation techniques and development in barley genomics together will pave a way for continuous development of barley cultivars having genes from more distant and alien backgrounds, widening the genetic base of existing cultivars.

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Chapter 6
Chickpea

C. Toker, B. Uzun, F.O. Ceylan, and C. Ikten

Abstract Chickpea (*Cicer arietinum* L.), the only cultivated species in the genus *Cicer* L., with $2n = 16$ chromosomes, is individually the most important pulse crop of the world and contributes significantly to the total food legume production. On the basis of plant type, pigmentation, flower and seed size and color, it is divided into two groups, *desi* and *kabuli*, both used in different culinary preparations as well as processed food products, thereby contributing as an important protein source in predominantly vegetarian diets. Tremendous efforts have already been put for the genetic improvement of chickpeas for yield, resistance to various stresses, and quality traits. However, unfortunately the selection process for improved yield and quality characteristics during domestication has resulted in a narrowing of the genetic variation of the cultivated chickpea. Not only do wild *Cicer* species consist of useful variation for morphological characteristics and protein content, but they also possess sources of resistance to biotic and abiotic stresses. Owing to these properties, breeders have tried to utilize these resources for transfer of desirable alleles from them into the cultivated species, mainly based on the information on gene pool concept. Nevertheless, success with distant hybridization has been variable and most of the successful crosses have been attempted only between the cultivated chickpea and the annual wild species of the primary and secondary gene pool while hybridization between the cultivated chickpea and perennial wild *Cicer* species has not been successful. Of late, genetic transformation and marker assisted breeding have also started yielding some results in alien gene transfer. This chapter reviews the sources for resistance to abiotic and biotic stresses in different *Cicer* species and also summarizes achievements and impacts of the introgression of alien genes from these wild species to the cultivated chickpea mainly via intraspecific hybridization.

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6.1 Introduction

The genus *Cicer* L. is a member of the tribe Cicereae, subfamily Faboideae or Papilionoideae, family Fabaceae or Leguminosae. It consists of 45 taxa including 9 annuals with the cultivated chickpea (*Cicer arietinum* L.) and 36 perennials (van der Maesen et al. 2007). The number of taxa in the genus *Cicer L.* has reached 49 taxa with the following perennials, *C. uludereensis* Donmez (Donmez 2011), *C. incisum* (Willd.) K. Maly subsp. *serpentinica* M.Ozturk & A. Duran, *C. floribundum* Fenzl. var. *amanicola* M. Ozturk & A. Duran and *C. heterophyllum* Contandr. et al. var. *kassianum* M. Ozturk & A. Duran (Ozturk et al. 2013). The genus *Cicer* L. possesses 40 species with a provisional wild species, *C. flexuosum* Lipsky, including 9 annual species with the cultivated chickpea and 32 perennial species (ILDIS 2012). *Cicer arietinum* L. is the only cultivated species in the genus *Cicer* L., with $2n = 16$ chromosomes, and it is a self-pollinated species due to its cleistogamic flowers (Cubero 1987). It is divided into two groups, "macrosperma" or *kabuli* and "microsperma" or *desi* (Muehlbauer and Singh 1987), on the basis of size and coloration of seeds and flowers, and pigmentation on plants. The "macrosperma" chickpeas have white flowers without pigments on their plants and have relatively larger and cream colored seeds. In contrast, the "microsperma" chickpeas have pink and blue flowers with pigments on their plant and small seed with different colors like brown, black, and green. The cultivated chickpea is a mutant derivative of *C. reticulatum* Ladizinsky, a wild progenitor species (Ladizinsky and Adler 1976a; Toker 2009). *C. reticulatum* Ladiz. originated from South-Eastern Turkey and Northern Syria (Ladizinsky and Adler 1976a; Zohary and Hopf 2000; Abbo et al. 2007; Toker 2009).

Selection process for better yield and quality characteristics during domestication have resulted in a drastic narrowing of the genetic variation of the cultivated chickpea (Toker 2009). Not only do wild *Cicer* species consist of useful variation for morphological characteristics (Robertson et al. 1995, 1997) and protein content (Ocampo et al. 1998), but they also possess sources of resistance to biotic (Di Vito et al. 1996; Collard et al. 2001; Ansari et al. 2004; Rubiales et al. 2004) and abiotic stresses (Singh et al. 1990, 1998; Croser et al. 2003; Toker 2005; Toker et al. 2007a, b; Canci and Toker 2009; Abbo et al. 2007). This chapter reviews the sources for resistance to abiotic and biotic stresses identified in *Cicer* species and achievements and impacts of the introgression of alien genes from these wild species to the cultivated chickpea via intraspecific hybridization.

6.2 Gene Pool Concept

According to International Legume Database and Information Service (2012), the genus *Cicer* L. possesses 41 species with a provisional wild species, *C. flexuosum* Lipsky, including 9 annual species with the cultivated chickpea and 32 perennial

Table 6.1 Annual wild *Cicer* species with variable morphological characteristics

Species	Stem length (cm)	Canopy width (cm)	Flower/pod per peduncle
C. arietinum L.	20–95	43–60	1 (2–3/7–9)[a]
C. bijugum K.H. Rech.	15–30	40–58	1 (2)[b]
C. chorassanicum (Bge) M. Pop.	5–15	6–15	1 (2)[b]
C. cuneatum Hochst. Ex Rich	40–60	7–13	1
C. echinospermum P.H. Davis	20–35	24–39	1
C. judaicum Boiss.	15–40	5–50	1 (2)[b]
C. pinnatifidum Jaub. & Sp.	10–40	9–33	1 (2)[b]
C. reticulatum Ladiz.	20–35	30–58	1
C. yamashitae Kitamura	10–30	8–13	1

[a]Generally single flower or pod per peduncle but some mutants bear multiple (up to nine) flowers or pods per peduncle
[b]Some accessions bear double flowers and pods

species (Tables 6.1 and 6.2). Based on crossability, karyotyping, morphological, physiological and molecular diversity studies as reviewed in this study, the wild *Cicer* species have been clustered on the basis of Harlan and de Wet's (1971) classical definition. The classification consists of primary (*C. arietinum*, *C. echinospermum*, and *C. reticulatum*), secondary (*C. pinnatifidum*, *C. bijugum*, and *C. judaicum*), and tertiary (*C. yamashitae*, *C. chrossanicum*, *C. cuneatum*, and perennial wild *Cicer* species) gene pools. Hybrids between *C. arietinum* and *C. pinnatifidum* (Mallikarjuna 1999), *C. arietinum* and *C. judaicum* (Verma et al. 1995), *C. arietinum* and *C. bijugum* (Mallikarjuna et al. 2007) were obtained via embryo rescue and tissue culture techniques. Abbo et al. (2011) produced some hybrids between *C. judaicum*×*C. bijugum*, and *C. cuneatum*×*C. canariense*. Clarke et al. (2011) obtained some hybrids between *C. arietinum* and *C. judaicum*, *C. arietinum*, and *C. pinnatifidum*, and reciprocal crosses. Therefore, on the basis of information on crossability, secondary gene pool covers *C. bijugum*, *C. judaicum*, and *C. pinnatifidum*. Tertiary gene pool contains the perennial wild *Cicer* species (Toker and Yadav 2010). Some of these, in general, possess more useful genes than those of annuals despite the fact that there is no report on successful hybridization between the cultivated chickpea and the perennial wild *Cicer* species.

6.3 Sources for Resistance to Abiotic Stresses

The growth and productivity of the cultivated chickpea in the world is adversely affected by several abiotic stresses. In the cultivated chickpea, the most common abiotic stresses in order are drought accompanying with heat in the Indian subcontinent, West and Central Asia, North and East Africa, Australia, and the Americas; cold (chilling and freezing) in Indian subcontinent, West and Central Asia, North and East Africa, and Australia; and nutrient imbalance including salinity in Indian subcontinent, Australia, and the Americas (Toker et al. 2007b). Efforts have been underway to identify the resistance sources to these stresses in the wild species.

Table 6.2 Wild perennial *Cicer* species and their stem length

Species[a]	Species[b]	Stem length (cm)
C. acanthophyllum Boriss.	*Cicer acanthophyllum* Boriss.	20–30
C. anatolicum Alef.	*Cicer anatolicum* Alef.	20–60
C. atlanticum Coss. ex Maire	*Cicer atlanticum* Maire	4–10
C. balcaricum Galushko	*Cicer balcaricum* Galushko	30–60
C. baldshuanicum Lincz.	*Cicer baldshuanicum* (Popov) Lincz.	30–40
C. canariense S. Guerra & Lewis	*Cicer canariense* A. Santos & G.P. Lewis	50–200
C. fedtschenkoi Lincz.	*Cicer fedtschenkoi* Lincz.	18–35
C. flexuosum Lipsky	*Cicer flexuosum* Lipsky	30–40
C. floribundum Fenzl.	–	15–30
C. floribundum Fenzl. var. *amanicola* M. Ozturk & A. Duran[d]	–	13–60
C. graecum Orph.	*Cicer graecum* Boiss.	30–40
C. grande Korotk.	*Cicer grande* (Popov) Korotkova	20–50
C. heterophyllum Contandr. vd.	–	40–70
C. heterophyllum Contandr. et al. var. *kassianum* M. Ozturk & A. Duran[d]	–	–
C. incanum Korotk.	*Cicer incanum* Korotkova	20–30
C. incisum K. Maly	*Cicer incisum* (Willd.) K. Maly	5–15
C. incisum (Willd.) K.Maly subsp. *serpentinica* M. Ozturk & A. Duran[d]	–	–
C. isauricum P.H. Davis	–	20–40
C. kermanense Bornm.	*Cicer kermanense* Bornm.	30–50
C. korshinskyi Linz.	*Cicer korshinskyi* Lincz.	50–80
C. laetum Rassulova & Sharipova	*Cicer laetum* Rassulova & Sharipova	50
C. macracanthum M. Pop.	*Cicer macracanthum* Popov	25–35
C. microphyllum Benth.	*Cicer microphyllum* Benth.	20–40
C. mogoltavicum Koroleva	*Cicer mogolatvicum* (Popov) A.S. Korol.	60–70
	Cicer mogoltavicum (Popov) A.S. Korol.	60–70
C. montbretti Jaub. & Sp.	*Cicer montbretii* Jaub. & Spach	40–60
C. multijugum van der Maesen	*Cicer multijugum* Maesen	10–30
C. nuristanicum Kitamura	*Cicer nuristanicum* Kitam.	25–40
C. oxyodon Boiss. & Hoh.	*Cicer oxyodon* Boiss. & Hohen.	25–55
C. paucijugum Nevski	*Cicer paucijugum* (Popov) Nevski	20–35
C. pungens Boiss.	*Cicer pungens* Boiss.	20–40
C. rassuloviae Lincz.	*Cicer rassulovae* Lincz.	–
C. rechingeri Podlech	*Cicer rechingeri* Podlech	40
C. songaricum Steph. ex. DC	*Cicer songaricum* DC.	25–40
C. spiroceras Jaub. & Sp.	*Cicer spiroceras* Jaub. & Spach	40–70
C. stapfianum K.H. Rech.	*Cicer stapfianum* Rech. f.	25
C. subaphyllum Boiss.	–	30–40
C. tragacanthoides Jaub. & Sp. var. *tragacanthoides*	*Cicer tragacanthoides* Jaub. & Spach	15–26
C. tragacanthoides Jaub. & Sp. var. *turcomanicum* Popov		11–35
C. uludereensis Donmez[c]		60–100

[a]van der Maesen et al. (2007)
[b]ILDIS (2012)
[c]Donmez (2011)
[d]Ozturk et al. (2013)

6.3.1 Drought

About 90 % of the cultivated chickpea in the world is grown under rainfed conditions (Kumar and Abbo 2001) where drought and heat stresses among abiotic stresses are the major constraints limiting its productivity (Singh et al. 1994; Ryan 1997; Croser et al. 2003; Toker et al. 2007b). Although annual wild *Cicer* species consist of adequate levels of resistance to drought as the cultivated chickpea, some accessions of perennial wild *Cicer* species including *C. anatolicum*, *C. microphyllum*, *C. montbretii*, *C. oxydon*, and *C. songaricum* were identified as highly resistance compared to annual wild species and the cultivated chickpea (Toker et al. 2007a). Canci and Toker (2009) selected some accessions of *C. reticulatum* and *C. pinnatifidum* as drought and heat resistant. Imtiaz et al. (2011) reported that two accessions each of *C. bijugum* and *C. reticulatum* recorded the highest yield in drought conditions in Syria (Table 6.3).

Table 6.3 Sources for resistance to abiotic stresses in *Cicer*

Species	Stress	Accession(s)	References
C. bijugum K.H. Rech.	Cold	ILWC 32, ILWC 62, ILWC 65, ILWC 66, ILWC 69, ILWC 70, ILWC 72, ILWC 73, ILWC 74, ILWC 76, ILWC 79	Singh et al. (1990, 1994, 1995, 1998)
	Cold	ILWC 32, ILWC 62, ILWC 63, ILWC 64, ILWC 65, ILWC 66, ILWC 67, ILWC 68, ILWC 69, ILWC 70, ILWC 72, ILWC 73, ILWC 74, ILWC 75, ILWC 76, ILWC 77, ILWC 79, ILWC 80, ILWC 83, ILWC 84, ILWC 177, ILWC 178, ILWC 194, ILWC 195, ILWC 209, ILWC 217, ILWC 220, ILWC 227, ILWC 228, ILWC 240, ILWC 241, ILWC 243	Robertson et al. (1995)
	Cold	AWC 1, AWC 2, AWC 3, AWC 4, AWC 5, AWC 6	Toker (2005)
	Drought	ILWC 34, ILWC 65	Imtiaz et al. (2011)
C. echinospermum P.H. Davis	Cold	ILWC 39, ILWC 179, ILWC 180, ILWC 181, ILWC 230, ILWC 235, ILWC 238, ILWC 239	Robertson et al. (1995)
	Cold	ILWC 39, ILWC 181	Singh et al. (1990, 1994, 1995, 1998)
	Cold	AWC 301, AWC 302, AWC 303, AWC 304, AWC 305, AWC 306, AWC 307	Toker (2005)
	Cold	ILWC 181, ILWC 235	Saeed et al. (2010)
C. pinnatifidum Jaub. & Sp.	Cold	ILWC 236	Singh et al. (1990, 1994, 1995, 1998)

(continued)

Table 6.3 (continued)

Species	Stress	Accession(s)	References
	Cold	ILWC 78, ILWC 88, ILWC 154, ILWC 157, ILWC 203, ILWC 204, ILWC 225, ILWC 236	Robertson et al. (1995)
	Cold	AWC 501, AWC 502, AWC 503	Toker (2005)
	Drought and heat	AWC 500	Canci and Toker (2009)
C. reticulatum Ladiz.	Cold	ILWC 81, ILWC 112, ILWC 113, ILWC 117, ILWC 139, ILWC 140, ILWC 141	Singh et al. (1990, 1994, 1995, 1998)
	Cold	ILWC 81, ILWC 104, ILWC 105, ILWC 106, ILWC 108, ILWC 109, ILWC 110, ILWC 111, ILWC 112, ILWC 113, ILWC 114, ILWC 115, ILWC 116, ILWC 117, ILWC 118, ILWC 120, ILWC 122, ILWC 123, ILWC 124, ILWC 125, ILWC 126, ILWC 127, ILWC 128, ILWC 129, ILWC 130, ILWC 131, ILWC 134, ILWC 135, ILWC 136, ILWC 137, ILWC 139, ILWC 140, ILWC 141, ILWC 142, ILWC 182, ILWC 183, ILWC 184, ILWC 216, ILWC 218, ILWC 219, ILWC 229, ILWC 231, ILWC 233, ILWC 242	Robertson et al. (1995)
	Cold	AWC 600, AWC 601, AWC 602, AWC 603, AWC 604, AWC 605, AWC 606, AWC 607, AWC 608, AWC 609, AWC 610, AWC 611, AWC 612, AWC 613, AWC 614	Toker (2005)
	Cold	ILWC 81, ILWC 106, ILWC 139	Saeed et al. (2010)
	Drought and heat	AWC 605, AWC 616, AWC 620, AWC 625	Canci and Toker (2009)
	Drought	ILWC 36, ILWC 116	Imtiaz et al. (2011)
C. canariense S. Guerra & Lewis	Drought and heat	ILWC 271	Toker unpublished
C. anatolicum Alef.		PI 561078, PI 383626, WG-14183	Toker et al. (2007b)
C. microphyllum Benht.		PI 532928	Toker et al. (2007b)
C. montbretii Jaub et. Sp.		WG-14189	Toker et al. (2007b)
C. oxydon Boiss. et Hoh.		PI 561103	Toker et al. (2007b)
C. songaricum Steph ex DC.		WG-4574	Toker et al. (2007b)

6.3.2 Suboptimal Temperatures

Suboptimal temperatures for chickpea can be categorized into two groups, i.e., low (cold) and high (heat) temperature stresses since the chickpea thrives well at temperatures between 15 and 29 °C (Imtiaz et al. 2011). Cold-related stresses were defined in terms of either chilling (between 0 and 12 °C) or freezing (below 0 °C) without snow cover (Wery et al. 1993; Toker et al. 2007a). Chilling temperatures below 10 °C, especially in the reproductive phase, negatively affect chickpea resulting in flower and pod abortions (Savithri et al. 1980; Srinivasan et al. 1999; Clarke and Siddique 2004; Nayyar et al. 2005a, b). Chickpea faces freezing stress during vegetative growth in West Asia and North Africa, Europe, and Central Asia, especially when sown in autumn or in early spring (Toker et al. 2007b).

Since favorable levels of cold tolerance are not available in the cultivated chickpea germplasm, wild *Cicer* species have been used as alternative sources for resistance to cold. Most accessions of *C. bijugum*, *C. echinospermum*, and *C. reticulatum* and some accessions of *C. pinnatifidum* were tolerant to cold (freezing) at seedling stage, and these accessions were found to be better than those accessions in the cultivated chickpea (Singh et al. 1990, 1994, 1995, 1998; Robertson et al. 1995; Toker 2005; Saeed et al. 2010).

Berger et al. (2012) evaluated 200 accessions of wild *Cicer* species for tolerance to chilling because there was little useful variation for chilling tolerance within the cultivated chickpea. *C. bijugum* and *C. judaicum* were more chilling tolerant species as compared to *C. reticulatum*, *C. pinnatifidum*, and *C. echinospermum*. They suggested that *C. echinospermum* has considerable potential as a robust donor of chilling tolerance since it is crossable with chickpea.

Chickpea is also sensitive to high temperatures, especially in the reproductive phase (Malhotra and Saxena 1993; Singh et al. 1994). Tolerance to heat has been identified, both in the cultivated (Dua 2001; Krishnamurthy et al. 2011) and wild species (*C. reticulatum* and *C. pinnatifidum*) of chickpea (Canci and Toker 2009) (Table 6.3).

6.3.3 Nutrient Imbalance

Deficiencies of macronutrients and micronutrients in chickpea not only reduce plant yield but also adversely affect nitrogen fixation. N and P deficiencies in chickpea have been reported to cause yield losses of 709,000 and 653,000 t per year in the world, respectively. Also, worldwide yield losses due to micronutrient deficiencies have been estimated at about 360,000 t per year (Ryan 1997). All accessions of wild *Cicer* species are tolerant to nutrient deficiencies (Toker unpublished data), while iron (Fe) deficiency is a serious problem in some germplasm of the cultivated chickpea (Toker et al. 2010b). Wild *Cicer* species can be evaluated for resistance to boron toxicity because it is a weak link and deserves attention.

6.3.4 Salinity

More than 800 million ha area in the world is affected by salt, through either salinity (397 million ha) or sodicity (434 million ha), which is over 6 % of the world's total land area (Munns 2005, 2010). Maliro et al. (2004) screened the wild relatives of *C. reticulatum* (eight accessions), *C. echinospermum* (three accessions), *C. bijugum* (six accessions), *C. judaicum* (one accession), and *C. pinnatifidum* (one accession) for tolerance to salinity, but they concluded that none of the wild accessions could be rated as tolerant to salinity. Nonetheless, further studies on salinity are required using more wild accessions (Maliro et al. 2004). In addition to drought, suboptimal temperatures, nutrient imbalance, and salinity, the cultivated chickpea also faces some region-specific minimal abiotic stresses (Toker et al. 2007b).

6.4 Sources for Resistance to Biotic Stresses

The growth, yield, and quality of the chickpea are also tremendously influenced by several fungal, bacterial, and viral diseases, insect pests, parasitic nematodes, and weeds. The prevalence of abiotic stresses and environmental conditions often affect the severity and occurrence of biotic stresses.

6.4.1 Ascochyta Blight

Ascochyta blight [*Ascochyta rabiei* (Pass.) Labr.] that can affect chickpea yield up to 100 % under favorable conditions is one of the major foliar diseases of chickpea in the world (Nene and Reddy 1987). Resistant sources for ascochyta blight were identified and have been detailed in Table 6.4. Singh et al. (1981) reported that one accession each of *C. pinnatifidum*, *C. montbretti*, and *C. judaicum* were highly resistant to ascochyta blight. Haware et al. (1992) also evaluated wild *Cicer* species for resistance to ascochyta blight and shortlisted resistant accessions. Singh and Reddy (1993) selected one accession each of *C. judaicum* and *C. pinnatifidum* as resistant in both field and greenhouse evaluations. Stamigna et al. (2000) found high level of double resistance to both ascochyta blight and fusarium wilt in accessions of *C. judaicum*, *C. bijugum*, and *C. pinnatifidum*. Collard et al. (2001) classified some accessions of *C. bijugum*, *C. echinospermum*, and *C. pinnatifidum* as resistant. In a different study, Collard et al. (2003a) also reported that an accession of *C. echinospermum* was resistant to ascochyta blight. Similarly Shah et al. (2005) identified 14 accessions of *C. bijugum*, 1 of *C. echinospermum*, 12 of *C. judaicum*, 15 of *C. pinnatifidum*, 1 of *C. reticulatum*, and 3 of *C. yamashitae* as resistant. Nguyen et al. (2005) found seven accessions of *C. judaicum* to be resistant to ascochyta blight while Pande et al. (2006) reported five resistant accessions of *C. judaicum* resistant

Table 6.4 Sources for resistance to ascochyta blight in *Cicer*

Species	Accession(s)	References
C. bijugum K.H. Rech.	ICCW 33, ICCW 34, ICCW 35, ICCW 36, ICCW 41, ICCW 42	Haware et al. (1992)
	ICCW 72	Haware et al. (1992)
	PI 458550, PI 458552	Kaiser et al. (1994)
	ILWC 62, ILWC 69, ILWC 70, ILWC 73	Singh et al. (1998)
	ILWC 76	Stamigna et al. (2000)
	ILWC 7, ILWC 64, ILWC 65, ILWC 69, ILWC 75, ILWC 76, ILWC 217, ILWC 241	Collard et al. (2001)
	ILWC 7, ILWC 8, ILWC 32, ILWC 34, ILWC 42, ICCW 47, ILWC 68, LR-051, W610149, W610150, 050689-0203, 050689-0301, 060689-0101, 090689-0301, 100689-0401	Shah et al. (2005)
C. chorassanicum (Bge) M. Pop.	PI 458553	Kaiser et al. (1994)
C. cuneatum Hochst. Ex Rich	PI 458554	Kaiser et al. (1994)
C. echinospermum P.H. Davis	ICCW 44	Haware et al. (1992)
	ILWC 245	Singh et al. (1998)
	ILWC 246, ILWC 245, PI 527930	Collard et al. (2001)
	PI 527930	Collard et al. (2003a, b)
	ICCW 44	Shah et al. (2005)
C. judaicum Boiss.	ICCW 75, ICCW 76, ICCW 89, ICCW 90, ICCW 97	Haware et al. (1992)
	ICC 17211, IG 69986, IG 70030, IG 70037, IG 70038	Pande et al. (2006)
	PI 458559	Kaiser et al. (1994)
	ILWC 255, ILWC 256	Singh et al. (1998)
	ILWC 186	Stamigna et al. (2000)
	ILWC 31, ILWC 45, ILWC 47, ILWC 45, ILWC 255, ILWC 272, ILWC 273, ILWC 274, ILWC 279, BMV 23-9, BMV 26-1, LR-126	Shah et al. (2005)
C. pinnatifidum Jaub. & Sp.	PI 458555, PI 458556, PI 510654, PI 51860	Kaiser et al. (1994)
	ICCW 11, ICCW 37, ICCW 38, ICCW 85, ICCW 86, ICCW 88, ICCW 94	Haware et al. (1992)
	ILWC 250, ILWC 251	Singh et al. (1998)
	ILWC 150	Stamigna et al. (2000)
	PI 518862	Collard et al. (2001)
	ICCW 38, ILCW 9+33, ILCW 19, ILWC 49, ILWC 51, ILCW 226, LR-198, 5119, 110785-401, 110785-0601, 120785-0101, 130785-0301, 040689-0603, 0406689-0703	Shah et al. (2005)
C. reticulatum Ladiz.	ILWC 113	Singh et al. (1998)
	ILWC 26, ILWC 130	Infantino et al. (1996)
	ILWC 104, ILWC 118, ILWC 119, ILWC 139	Collard et al. (2001)
	ILWC 17+21	Shah et al. (2005)
C. yamashitae Kitamura	ILWC 3, ILWC 214, ILWC 215	Shah et al. (2005)
C. canariense S. Guerra & Lewis	PI 557453	Kaiser et al. (1994)

Table 6.5 Sources for resistance to fusarium wilt in *Cicer*

Species	Races/isolate origin	Accession(s)	References
C. bijugum K.H. Rech.	Isolate from India	ICCW 72	Haware et al. (1992)
	Race 0 and 5	PI 458550, PI 458552	Kaiser et al. (1994)
	Isolate from Italy	ILWC 64, ILWC 71, ILWC 73, ILWC 76, ILWC 80, ILWC 83	Infantino et al. (1996)
	Isolate from Syria	ILWC 62, ILWC 65, ILWC 66, ILWC 69, ILWC 70, ILWC 73, ILWC 74, ILWC 76, ILWC 79	Singh et al. (1998)
C. chorassanicum (Bge) M. Pop.	Race 0	PI 458553	Kaiser et al. (1994)
C. cuneatum Hochst. Ex Rich	Race 0 and 5	PI 458554	Kaiser et al. (1994)
	Isolate from Syria	ILWC 187, ILWC 232	Singh et al. (1998)
C. echinospermum P.H. Davis	Isolate from India	ICCW 44	Haware et al. (1992)
	Isolate from Syria	ILWC 39, ILWC 245	Singh et al. (1998)
C. judaicum Boiss.	Race 0 and 5	PI 458559	Kaiser et al. (1994)
	Isolate from Italy	ILWC 186	Infantino et al. (1996)
	Isolate from India	–	Nene and Haware (1980)
	Isolate from Syria	ILWC 46, ILWC 189, ILWC 255, ILWC 256	Singh et al. (1998)
C. pinnatifidum Jaub. & Sp.	Race 0 and 5	PI 458555, PI 458556, PI 510654, PI 51860	Kaiser et al. (1994)
	Isolate from Syria	ILWC 171, ILWC 250, ILWC 251	Singh et al. (1998)
C. reticulatum Ladiz.	Isolate from Italy	ILWC 26, ILWC 130	Infantino et al. (1996)
	Isolate from Syria	ILWC 81, ILWC 112, ILWC 117, ILWC 139, ILWC 140, ILWC 141	Singh et al. (1998)
C. canariense S. Guerra & Lewis	Race 0	PI 557453	Kaiser et al. (1994)

to ascochyta blight. Sources of resistance to ascochyta blight show different reaction from one region or country to the other regions due to different pathotypes (Table 6.5).

In Israel, Frenkel et al. (2007) isolated two distinct pathogens from *C. judaicum* and identified the pathogens as *Phoma pinodella* and *Didymella rabiei*. They reported that *P. pinodella* infected *Pisum sativum*, *P. fulvum*, *C. judaicum*, *C. arietinum*, *C. reticulatum*, *C. pinnatifidum*, and *C. bijugum*, while *D. rabiei* was virulent to all above *Cicer* species, but not *P. sativum* and *P. fulvum*. It was suggested that *C. judaicum* may serve as an alternative host to ascochyta pathogens that endanger chickpea and possibly other crops and wild species (Frenkel et al. 2007). It can therefore be suggested that accessions of wild relatives of chickpea should be selected according to the pathotypes prevalent in the target region.

Table 6.6 Sources for resistance to botrytis gray mold and the other diseases in *Cicer*

Species	Biotic stress	Accession(s)	Reference
C. bijugum K.H. Rech.	Botrytis gray mold	ICCW 41, ICCW 42, ICCW 91	Haware et al. (1992)
	Collard rot	ICCW 72	Haware et al. (1992)
	Botrytis gray mold	IG 69981, IG 70023, IG 70006	Pande et al. (2006)
	Botrytis gray mold	ILWC 240	Isenegger et al. (2011)
C. echinospermum P.H. Davis	Phytophthora root rot	ILWC 245, ILWC 246	Knights et al. (2008)
C. judaicum Boiss.	Botrytis gray mold	ICC 17194, ICC 17205, ICC 17149, ICC 17148, ICC 17204, IG 69977, IG 70033, IG 72931, IG 72932, IG 17150, IG 69959, IG 69969, IG 70032, IG 70038, ICC 17151, ICC 17190, ICC 17192, ICC 17195, IG 69943, IG 69997, IG 69998, IG 70034, IG 70037	Pande et al. (2006)
C. reticulatum Ladiz.	Botrytis gray mold	IG 72959, IG 72933, IG 72941	Pande et al. (2006)

6.4.2 *Fusarium Wilt*

Fusarium wilt caused by *Fusarium oxysporum* Schlechtend. Fr. f. sp. *ciceris* (Padwick) Matua & K. Sato is the other major biotic constraint to chickpea production in the world (Nene and Haware 1980). *C. bijugum*, *C. chorassanicum*, *C. cuneatum*, *C. echinospermum*, *C. judaicum*, *C. microphyllum*, *C. pinnatifidum*, *C. reticulatum*, and *C. yamashitae* were screened for resistance to fusarium wilt (Nene and Haware 1980) and only the accessions of *C. judaicum* were found as resistant (Table 6.6). Kaiser et al. (1994) identified resistance to race 0 and 5 in some accessions of *C. bijugum*, *C. cuneatum*, *C. judaicum*, and *C. pinnatifidum*. Infantino et al. (1996) found six accessions of *C. bijugum*, one accession of *C. judaicum* and two accessions of *C. reticulatum* free from fusarium wilt damage. Similarly, in other studies, Singh et al. (1994, 1995, 1998) reported many other wild chickpea species as resistant: ten accessions of *C. bijugum*, two of *C. cuneatum*, two (one of them as tolerant) of *C. echinospermum*, two of *C. judaicum*, three of *C. pinnatifidum,* and five accessions of *C. reticulatum*. Kaiser et al. (1994) identified an accession of *C. canariense* (PI 557453) as resistant to *F. oxysporum* f. sp. *ciceris* race 0 and 5 in growth chamber and microplot.

6.4.3 *Botrytis Gray Mold*

Botrytis gray mold caused by *Botrytis cinera* Pers. ex. Fr., the anamorph of *Botryotinia fuckeliana* (de Bary) Whetzel, causes severe damage to chickpea if the conditions are favorable (Pande et al. 2006). Resistance to botrytis gray mold was

reported in *C. pinnatifidum* (Singh et al. 1982), and *C. bijugum* (Haware et al. 1992). Among the annual wild *Cicer* species, Pande et al. (2006) evaluated 148 wild accessions chickpea for resistance to botrytis gray mold, and identified 3 accessions of *C. bijugum*, 23 accessions of *C. judaicum*, and 3 accessions of *C. reticulatum* as sources of resistance (Table 6.7).

Table 6.7 Sources for resistance to important insect pests and other biotic stresses in *Cicer*

Species	Biotic stress	Accession(s)	References
C. bijugum K.H. Rech.	Crenate broomrape	C 500	Rubiales et al. (2004)
	Cyst nematode	ILWC 217	Di Vito et al. (1996)
	Cyst nematode	ILWC 62, ILWC 66, ILWC 65, ILWC 69, ILWC 70, ILWC 73, ILWC 74, ILWC 76, ILWC 76, ILWC 79	Singh et al. (1998)
	Leaf miner	ILWC 66, ILWC 72, ILWC 79	Singh et al. (1998)
	Pod borer	IG 70003, IG 70022, IG 70016, IG 70013, IG 70012, IG 70010, IG 70001, IG 70018, IG 70002, ICC 17257, IG 70002	Sharma et al. (2005b)
	Seed beetle	ILWC 32, ILWC 62, ILWC 65, ILWC 69, ILWC 70, ILWC 72, ILWC 73, ILWC 74, ILWC 79	Singh et al. (1994, 1998)
C. cuneatum Hochst. Ex Rich	Leaf miner	ILWC 40, ILWC 87	Singh et al. (1998)
	Leaf miner	ILWC 187, ILWC 232	Singh et al. (1998)
	Pod borer	IG 69979	Sharma et al. (2005b)
C. echinospermum P.H. Davis	Leaf miner	ILWC 39, ILWC 245	Singh et al. (1998)
	Seed beetle	ILWC 39, ILWC 181, ILWC 245	Singh et al. (1994, 1998)
C. judaicum Boiss.	Cyst nematode	ILWC 46, ILWC 189, ILWC 255, ILWC 256	Singh et al. (1998)
	Leaf miner	ILWC 44, ILWC 45, ILWC 46, ILWC 56, ILWC 57, ILWC 58, ILWC 61, ILWC 95, ILWC 96, ILWC 101, ILWC 102, ILWC 103, ILWC 161, ILWC 175, ILWC 186, ILWC 187, ILWC 188, ILWC 189, ILWC 191, ILWC 192, ILWC 193, ILWC 196, ILWC 198, ILWC 199, ILWC 201, ILWC 202, ILWC 205, ILWC 206, ILWC 207	Singh and Weigand (1994)
	Leaf miner	ILWC 46, ILWC 189, ILWC 255, ILWC 256	Singh et al. (1998)
	Pod borer	IG 70032, IG 70033, IG 70038, IG 72931	Sharma et al. (2005b)
	Seed beetle	ILWC 46, ILWC 189	Singh et al. (1994, 1998)

(continued)

Table 6.7 (continued)

Species	Biotic stress	Accession(s)	References
C. pinnatifidum Jaub. & Sp.	Crenate broomrape	C 337	Rubiales et al. (2004)
	Cyst nematode	ILWC 49, ILWC 212, ILWC 213, ILWC 226, ILWC 250, ILWC 252	Di Vito et al. (1996)
	Cyst nematode	ILWC 250	Singh et al. (1998)
	Leaf miner	ILWC 60, ILWC 82, ILWC 100, ILWC 225	Singh and Weigand (1994)
	Leaf miner	ILWC 171, ILWC 250, ILWC 255	Singh et al. (1998)
	Pod borer	IG 69948, IG 70039	Sharma et al. (2005b)
	Seed beetle	ILWC 171, ILWC 236	Singh et al. (1994, 1998)
C. reticulatum Ladiz.	Crenate broomrape	C 553	Rubiales et al. (2004)
	Cyst nematode	ILWC 119	Di Vito et al. (1996)
	Cyst nematode	ILWC 81, ILWC 112, ILWC 117, ILWC 139, ILWC 140, ILWC 141	Singh et al. (1998)
	Leaf miner	ILWC 81	Singh and Weigand (1994), Singh et al. (1998)
	Pod borer	IG 72933, IG 72934, IG 72936, IG 72953	Sharma et al. (2005a)
	Seed beetle	ILWC 81, ILWC 112, ILWC 113, ILWC 117	Singh et al. (1994, 1998)
C. canariense S. Guerra & Lewis	Crenate broomrape	–	Rubiales et al. (2004)
	Pod borer	ICC 17202	Sharma et al. (2006)
C. macracanthum M. Pop.	Crenate broomrape	–	Rubiales et al. (2004)
C. microphyllum Benth.	Pod borer	ICC 17146, ICC 17230, ICC 17234, ICC 17236, ICC 17240, ICC 17243, ICC 17244, ICC 17248	Sharma et al. (2006)
C. oxydon Boiss. et Hoh.	Crenate broomrape	C 336	Rubiales et al. (2004)
C. songaricum Steph ex DC.	Crenate broomrape	–	Rubiales et al. (2004)

6.4.4 Collar Rot

Collar rot (*Scerotium rolfsii* Sacc.) is one of the known diseases of chickpea (Haware et al. 1992), and sources of moderate resistance to collar rot in the wild relative, *Cicer bijugum*, are available (Table 6.7).

Table 6.8 Successful F_1 hybrids and F_2 populations obtained from crosses involving wild *Cicer* species

Crosses	References
C. arietinum × *C. reticulatum*	Ladizinsky and Adler (1976a, b), Jaiswal et al. (1986), Singh and Ocampo (1993), Simon and Muehlbauer (1997), Singh and Ocampo (1997), Winter et al. (1999), Santra et al. (2000), Winter et al. (2000), Rajesh et al. (2002), Tekeoglu et al. (2002), Pfaff and Kahl (2003), Abbo et al. (2005), Singh et al. (2005), Madrid et al. (2008)
C. arietinum × *C. echinospermum*	Singh and Ocampo (1993), Simon and Muehlbauer (1997), Singh and Ocampo (1997), Collard et al. (2003a, b), Ahmad and Slinkard (2004)
C. reticulatum × *C. arietinum*	Singh and Ocampo (1993)
C. echinospermum × *C. arietinum*	Singh and Ocampo (1993), Ahmad and Slinkard (2004)
C. reticulatum × *C. echinospermum*	Iruela et al. (2002)

6.4.5 Leaf Blight

Leaf blight [*Colletotrichum capsici* (Syd.) E.J. Butler & Bisby], which may cause up to 100 % mortality in chickpea and its wild relatives, is also one of the important devastating diseases (Nene and Haware 1980). *C. judaicum* was found as resistant to both, fusarium wilt and the leaf blight. A collection no. 188 of *C. pinnatifidum*, showed 73.6 % mortality, while all other species showed 100 % mortality (Nene and Haware 1980). However, this cannot be treated as a strong source of resistance to this disease, and therefore more detailed screening studies are required.

6.4.6 Pod Borer

The pod borer (*Helicoverpa armigera* Hubner), a voracious feeder, is one of the most important pests of chickpea in many parts of the world, and causes considerable yield losses (Sharma et al. 2007). Although moderate level of resistance to pod borer has been found in the cultivated chickpea, sources possessing high level of resistance to this pest in wild *Cicer* species are required. High level of resistance to pod borer were detected in some accessions of wild relatives of chickpea, i.e., *Cicer bijugum*, *C. pinnatifidum, C. cuneatum*, *C. judaicum*, *C. pinnatifidum*, and *C. reticulatum* (Table 6.8; Sharma et al. 2005a, b). Sharma et al. (2005a) pointed out that some accessions of *C. reticulatum* namely, IG 69960, IG 72934, and IG 72936 were comparatively more resistant to pod borer than those of the cultivated chickpeas or other annual wild accessions at the vegetative and reproductive stages. Some of the accessions belonging to *C. canariense* and *C. microphyllum* (Table 6.7) were also found resistant to pod borer (Sharma et al. 2006).

6.4.7 Leaf Miner

In the Mediterranean area, leaf miner (*Liriomyza cicerina* Rond.) is one of the most important insect pests feeding on the leaves of chickpea (Singh and Weigand 1994; Singh et al. 1998; Toker et al. 2010a). Chickpea leaf miner causes yield reductions that depend on infestation level, chickpea genotype, the environment and whether crops are spring or winter-sown; yield losses can reach up to 40 % (Reed et al. 1987). Resistance level to leaf miner in wild chickpea species is higher than that is the cultivated chickpeas (Singh et al. 1998). Leaf miner resistance sources in annual wild species including *C. bijugum*, *C. echinospermum*, *C. pinnatifidum*, *C. judaicum*, *C. chorassanicum*, and *C. reticulatum* (Table 6.8) have been reported in several studies (Singh and Weigand 1994; Singh et al. 1994, 1998; Robertson et al. 1995). However, none of the annual wild *Cicer* species reported as resistant in Table 6.7 was free from leaf miner damage while a mutant of *C. reticulatum* having multipinnate leaves, tiny leaflets, and dark pigmentation on plant was free from leaf miner damage under heavy infestation conditions compared to susceptible and improved lines by ICARDA (Fig. 6.1).

6.4.8 Seed Beetles (Bruchid)

The seed-beetles in the genus *Callosobruchus* Pic. (Coleoptera: Bruchidae) are economically important pests of stored pulse crops (Reed et al. 1987; Sharma et al. 2007). These cosmopolitan pests can cause considerable crop losses in short times (Sharma et al. 2007). As seen in Table 6.8, some accessions of *C. bijugum*, *C. echinospermum*, *C. judaicum*, *C. pinnatifidum*, and *C. reticulatum* were found as resistant to bruchid (Singh et al. 1994, 1998).

6.4.9 Nematodes

The most common nematodes affecting chickpea are *Meloidogyne* species, *Heterodera ciceri*, *Rotylenchulus reniformis*, and *Pratylenchus* species (Greco 1987; Thompson et al. 2012). Some of these nematodes can not only reduce crop yield up to 50 % (Greco 1987; Knights et al. 2008), but also adversely affect nitrogen fixation and transmit viruses (Greco 1987). The root lesion nematodes, *Pratylenchus thornei* Sher and Allen and *P. neglectus* (Rensch) Filipjev & Schuurmans Stekhoven, are widely distributed in the Australian, and biological or seed yield of the chickpea were reduced by root lesion nematodes in the range 25–60 % (Thompson et al. 2012).

Cyst nematode (*Heterodera ciceri* Vovlas, Greco et Di Vito) is an important yield reducer among the above nematodes (Greco et al. 1984). Resistance to cyst nematode was detected in accessions of *C. bijugum*, *C. pinnatifidum*, and *C. reticulatum*

Fig. 6.1 Comparison of a mutant of *C. reticulatum* (*above right*) to the cultivated chickpeas having simple, normal, and multipinnate leaves for resistance to leaf miner

(Di Vito et al. 1996). Three accessions of *C. bijugum* were found as resistant to cyst nematode (Di Vito and Vovlas 1990). In another study, Singh et al. (1989) selected 21 accessions of *C. bijugum* as resistant to *Heterodera ciceri* Vovlas, Greco *et* Di Vito, while all accessions of other annual wild *Cicer* species were susceptible or highly susceptible to *H. ciceri*. Resistance level of annual wild *Cicer* species for cyst nematode was confirmed by successive studies (Di Vito et al. 1996; Singh et al. 1994, 1998).

6.5 Parasitic Weeds and Herbicide Resistance

Crenate broomrape (*Orobanche crenata* Forsk.) is a major constraint in the production of faba bean (*Vicia faba* L.), pea (*Pisum sativum* L.), lentil (*Lens culinaris* Medik.), some forage legumes including vetch (*Vicia sativa* L.), and autumn-sown chickpeas

in the Mediterranean region and West Asia (Linke et al. 1991; Rubiales et al. 2004). Rubiales et al. (2004) screened wild *Cicer* species for resistance to crenate broomrape and found that all the accessions of *C. bijugum*, *C. echinospermum*, *C. judaicum*, and *C. pinnatifidum* were highly resistant, whereas most of the accessions of *C. reticulatum* were highly resistant in pot and Petri dish experiments (Table 6.7). As seen in Table 6.5, *C. canariense*, *C. macracanthum*, *C. multijugum*, *C. oxyodon*, and *C. songaricum* were found as resistant to crenate broomrape in field screenings (Rubiales et al. 2004).

Yield losses due to weeds in chickpea are significant (Yenish 2007) because of its slow initial growth during its seedling stage. Development/identification of herbicide-resistant chickpea cultivars is therefore top priority to solve weed problem. Ceylan and Toker (2006) identified some accessions of *C. reticulatum* as tolerant to post-emergence herbicides. Toker et al. (2012) recorded a mutant of *C. reticulatum* as resistant to herbicide, imidazilonone. Also, some accessions of *C. reticulatum* and *C. pinnatifidum* were able to recover after glyphosate application, but not the cultivated and the other annual wild *Cicer* species (Toker unpublished data).

6.6 Protective Substances

Wild *Cicer* species are resistant to abiotic and biotic stresses due to the fact that they are able to produce some kind of substances and secondary metabolites. From the roots of *Cicer judaicum*, Stevenson and Veitch (1996) isolated different isoflavenes, viz., three new isoflav-3-enes, 7-hydroxy-2′-methoxy-4′,5′-methylenedioxyisoflav-3-ene (judaicin), judaicin 7-*O*-glucoside, and judaicin 7-*O*-(6′-*O*-malonylglucoside), and the known pterocarpans, maackiain 3-*O*-glucoside and maackiain 3-*O*-(6′-*O*-*malonylglucoside*). Veitch and Stevenson (1997) isolated 2-methoxyjudaicin from roots of *C. bijugum*. Stevenson and Veitch (1998a) also detected the pterocarpans medicarpin and maackiain and the isoflavonoids biochanin A and formononetin in the roots of all of 15 *Cicer* species including *C. bijugum*, *C. judaicum* and *C. pinnatifidum*. Cicerfuran was found to be more potent than both maackiain and medicarpin, which are known antifungal phytoalexins in *Cicer* (Stevenson and Veitch 1998b). Stevenson and Haware (1999) postulated that there was a negative association between high concentrations of maackiain and botrytis gray mold in resistant accessions of *C. bijugum* when compared to susceptible species, *C. arietinum*, *C. echinospermum*, and *C. reticulatum*. They indicate that maackiain might be an important component in botrytis gray mold resistance in *C. bijugum* and that the resistance is enhanced in the presence of the pathogen. Aslam et al. (2009) studied antibacterial and antifungal activity of cicerfuran and related 2-arylbenzofurans and stilbenes against two species of bacteria, *Bacillus subtilis* and *Pseudomonas syringae*, and four species of filamentous fungi, *Aspergillus niger*, *Botrytis cinerea*, *Cladosporium herbarum*, and *Monilinia aucuporiae*. They concluded that cicerfuran showed antimicrobial activity. Simmonds and Stevenson (2001) concluded that the isoflavonoids, especially maackiain and judaicin, could play a role in decreasing the susceptibility of *Cicer* to attack by *H. armigera*.

6.7 Interspecific Hybridization

Interspecific hybridization in chickpea is the crossing of two species of the genus *Cicer* L. in order to transfer of useful characters or genes from wild species to the cultivated chickpea (Muehlbauer et al. 1994; Croser et al. 2003). Although a number of studies have been carried out to facilitate the useful gene transfer from wild *Cicer* species to the cultivated chickpea or vice versa (Table 6.8), hybridization between the cultivated chickpea and perennial wild *Cicer* species has not been successful. Some accessions of *C. bijugum*, *C. judaicum*, and *C. pinnatifidum* possess resistance to biotic and abiotic stresses (Tables 6.3, 6.4, 6.5, 6.6, and 6.7). However, hybridization barriers affect successful hybridization and most of these are post-zygotic barriers because zygote has been found to be formed successfully after the pollinations (Ahmad et al. 1988; Croser et al. 2003; Ahmad and Slinkard 2004; Clarke et al. 2006; Mallikarjuna et al. 2011). According to available literature, the first attempt to obtain hybrid was done by Ladizinsky and Adler (1976a, b). To date, crosses between *C. arietinum* and *C. reticulatum* and their reciprocals produced completely successful hybrids, while crosses between *C. arietinum* and *C. echinospermum* or vice versa produced partly successful hybrids (Table 6.8). These interspecific hybridizations have resulted in transferring of alien genes for several agronomically important traits.

6.7.1 Yield and Yield Components

Jaiswal et al. (1986) indicated that yield of the cultivated chickpea could be improved by the introgression of genes from *C. reticulatum*. Singh and Ocampo (1993) not only found a heterosis (hybrid vigor) of 28–153 % in the F_1s of crosses between the cultivated chickpea and *C. echinospermum* and *C. reticulatum*, but they also found fruitful transgressive segregants for yield in the F_2 generation. Similarly, heterosis of 13 % in plant height and 191 % in canopy width in the F_1 generation of crosses between the cultivated chickpea and *C. reticulatum,* and heterosis of 196 % in canopy width in the same generation of crosses between *C. reticulatum* and the cultivated chickpea was found (Fig. 6.2). The percentages of heterosis for pods and seeds per plant for the crosses between the cultivated chickpea and *C. reticulatum* were observed to be 204 % and 157 %, respectively (Fig. 6.3). Heterosis values for pods and seeds per plant in the crosses between *C. reticulatum* and the cultivated chickpea were 179 and 29, respectively. Similarly, heterosis values for biological and seed yields of the crosses between *C. reticulatum* and the cultivated chickpea were counted as 8 % and 1 %, respectively; while these values for biological and seed yields of the crosses between the cultivated chickpea and *C. reticulatum* were found as 111 % and 148 %, respectively. For harvest index, heterosis was a little bit lower in the crosses between the cultivated chickpea and *C. reticulatum* (23 %) than that of crosses between *C. reticulatum* and the cultivated chickpea (27 %) as shown

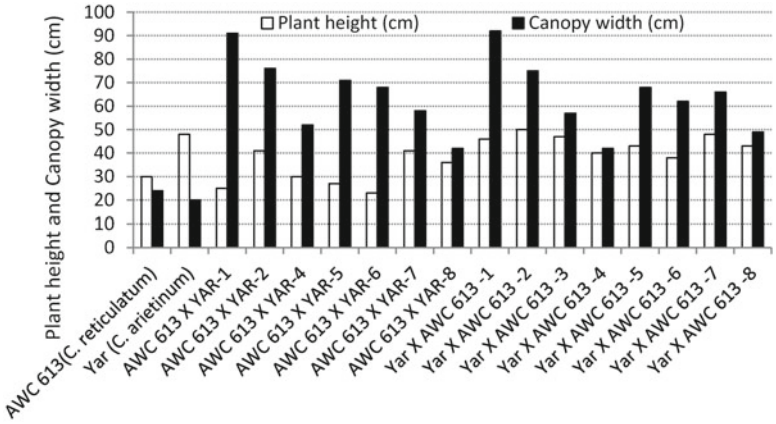

Fig. 6.2 Plant height and canopy width of F_1 crosses between *C. reticulatum* (♀) and the cultivated chickpea cv. Yar (♂), and between the cultivated chickpea cv. Yar (♀) and *C. reticulatum* (♂) as compared with their parents

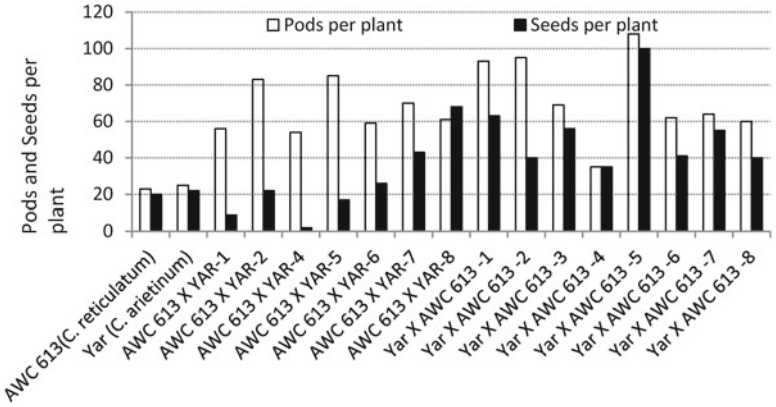

Fig. 6.3 Pods and seeds per plant of F_1 crosses between *C. reticulatum* (♀) and the cultivated chickpea cv. Yar (♂), and between the cultivated chickpea cv. Yar (♀) and *C. reticulatum* (♂) as compared with their parents

in Fig. 6.4. Heterosis values for 100-seed weight was negative in the crosses between *C. reticulatum* and the cultivated chickpea, while it was not recorded in the crosses between the cultivated chickpea and *C. reticulatum*. Transgressive segregants for yield (Fig. 6.5) and yield components (Figs. 6.5 and 6.6) in F_2 generation were noticed. Some plants in F_2 generation of crosses between double-flowered cultivated chickpea and *C. reticulatum* produced multiple flowers with a high degree of penetrance and expressivity (Toker unpublished data).

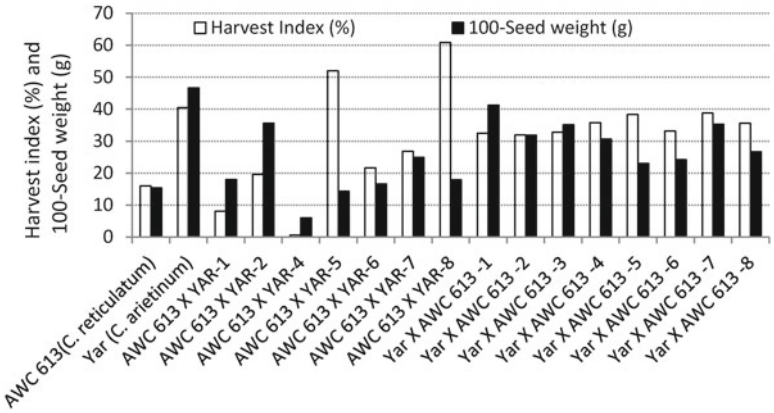

Fig. 6.4 Harvest index and 100-seed weight of F_1 crosses between *C. reticulatum* (♀) and the cultivated chickpea cv. Yar (♂), and between the cultivated chickpea cv. Yar (♀) and *C. reticulatum* (♂) as compared with the their parents

Fig. 6.5 Field performance of an F_2 population (*right row*) obtained from the crosses between the cultivated chickpea (*left row*) and *C. echinospermum* (*central row*) to their parents

As seen in Figs. 6.2, 6.3, and 6.4, performance of the F_1s of crosses between the cultivated chickpea (♀) and *C. echinospermum* (♂) or *C. reticulatum* (♂) was in general higher than those of the intraspecific crosses including those between *desi* and *kabuli* chickpea and vice versa (Singh et al. 1984).

Fig. 6.6 A line (*center*) selected in F_2 with two to three seeds/pod in crosses between *C. reticulatum* (♀, *left*) and the cultivated chickpea cv. Yar (♂, *right*)

Singh and Ocampo (1997) made crosses between *C. arietinum* (ILC 482)×*C. echinospermum* (ILWC 179) and *C. arietinum* (ILC 482)×*C. reticulatum* (ILWC 124) as well as their reciprocals and visually recorded heterosis in the F_1s. Further, some F_2 plants had two to three times higher seed yield than that of the cultivated chickpea (ILC 482). Some lines in F_7 generation had not only high yield (up to 39 %) but were also free of any known undesirable traits from the wild species (Singh and Ocampo 1997). These studies indicated that yield and yield components could be improved in cultivated chickpea by the introgression of genes from *C. reticulatum* and use of cultivated chickpea as female parent can give expected results in improvement of yield through introgression of alien genes from wild species.

6.7.2 Resistance to Abiotic and Biotic Stresses

The F_7 lines obtained from an interspecific cross between cultivated chickpea and *C. reticulatum* had a high degree of resistance to wilt, foot rot and root rot diseases, and recorded 6.1–17.0 % seed yield increase over the best check cultivars, GPF2 (Singh et al. 2005). Similarly, Knights et al. 2008 reported that the derivatives from crosses between the cultivated chickpea and *C. echinospermum* showed resistance to phytophthora root rot and *C. echinospermum* could be the best of the source for resistance to *P. medicaginis*.

Thompson et al. (2012) reported that *C. bijugum*, *C. echinospermum* and *C. reticulatum* were generally more resistant than those of the commercial chickpea cultivars grown in Australia to root-lesion nematodes (*P. thornei* and *P. neglectus*). The resistant accessions of *C. reticulatum* and *C. echinospermum* were crossed and topcrossed with *desi* chickpea cultivars and resistant F_4 lines were obtained (Thompson et al. 2012).

6.8 Molecular Markers

Molecular markers has been routinely employed for genetic mapping in interspecific (*C. arietinum* × *C. reticulatum, C. arietinum* × *C. echinospermum*) populations (Gaur and Slinkard 1990a, b; Kazan et al. 1993; Simon and Muehlbauer 1997) and these facilitated early framework for molecular mapping and marker assisted selection (MAS). The wild species *C. reticulatum* has been used as one of the parents in development of mapping populations (e.g., F_2 or RIL) (Winter et al. 1999, 2000; Santra et al. 2000; Cobos et al. 2005) and further involved in elucidation of chromosomal location of important QTL's. For example, alien genes/QTL controlling resistance to ascochyta blight in a wild species of chickpea were first associated with molecular markers by Collard et al. (2003a, b). Two QTL resistant to ascochyta blight have been identified in the background of wild species *C. echinospermum* and localized on LG4. Though wild *Cicer* species carried several important traits, they were scarcely utilized in mapping of these traits using molecular markers. For example, ascochyta blight resistance is an important trait for stable chickpea production. However, only a wild species has been utilized as resistant parent in most of studies conducted for marker trait association analysis (Santra et al. 2000; Tekeoglu et al. 2000, 2002; Rakshit et al. 2003; Collard et al. 2003a, b; Cho et al. 2004; Cobos et al. 2006, 2009; Iruela et al. 2006; Aryamanesh et al. 2010). Similarly, fusarium wilt resistance has been identified in several accessions of wild species. However, it has been utilized as susceptible parent (Infantino et al. 1996; Winter et al. 2000; Tekeoglu et al. 2000). The studies conducted to map the alien genes using the molecular in the background of wild species have been listed in Table 6.9.

Table 6.9 Molecular mapping using wild relatives in the genus *Cicer* L

Wild species	Trait/map	Type of markers	Reference
C. reticulatum	Linkage/transcript map	2,496 ESTs, 125 EST-SSRs, 151 ITPs, 109 ESTPs, 102 SNPs	Choudhary et al. (2012)
C. reticulatum	Transcriptome analysis	446 SNPs, 561 SSRs	Jhanwar et al. (2012)
C. reticulatum	Second generation genetic map	625 CKAMs, 314 TOG-SNPs, 389 marker loci	Hiremath et al. (2012)
C. reticulatum	Genetic map	1,344 SSR, 675 DArT	Thudi et al. (2011)
C. reticulatum	Genetic map	135 STMSs, 3 SCARs, 1 ASAP, ISSR, RAPD	Millan et al. (2010)
C. reticulatum	Plant growth habit, ascochyta blight resistance, days to flowering	Microsatellites	Aryamanesh et al. (2010)

(continued)

Table 6.9 (continued)

Wild species	Trait/map	Type of markers	Reference
C. reticulatum	Genetic map	311 SSR, 71 SNPs	Nayak et al. (2010)
C. reticulatum	Linkage map	6 RGAs, 5 CAPS, 2 dCAPS	Palomino et al. (2009)
C. reticulatum	Rust resistance	STMS	Madrid et al. (2008)
C. reticulatum	Linkage map, QTL, Ascochyta blight	RAPD, ISSR, STMS	Cobos et al. (2006)
C. reticulatum	Genetic map, 4 QTLs for beta-carotene, 1 QTL for lutein and 3 QTLs for seed weight	91 STMS, 2 CytP450	Abbo et al. (2005)
C. reticulatum	Linkage map, Fusarium wilt resistance	DAF, SCAR	Benko-Iseppon et al. (2003)
C. reticulatum	Ascochyta blight resistance, QTL1	DAF, STMS	Rakshit et al. (2003)
C. reticulatum, *C. echinospermum*, *C. bijugum*	Ascochyta blight resistance	RAPD, ISSR	Collard et al. (2003a)
C. reticulatum	Genetic map	STMS, RGA	Tekeoglu et al. (2002)
C. reticulatum	Genetic map	RGA, RFLP, CAPS	Huettel et al. (2002)
C. reticulatum	Linkage map	RGA	Rajesh et al. (2002)
C. reticulatum, *C. echinospermum*	Linkage map, 3 QTLs	RAPD	Banerjee et al. (2001)
C. reticulatum	Ascochyta blight resistance, linkage map, QTL1, QTL2	RAPD, ISSR	Santra et al. (2000)
C. reticulatum	Linkage map, resistance for Fusarium wilt	118 STMSs, 96 DAFs, 70 AFLPs, 37 ISSRs, 17 RAPDs, 8 Isozymes, 3 cDNAs, 2 SCARs	Winter et al. (2000)
C. reticulatum, *C. echinospermum*	Genetic map	120 STMS	Winter et al. (1999)
C. reticulatum	Linkage map	27 Isozymes, 10 RFLPs, 45 RAPDs	Simon and Muehlbauer (1997)
C. echinospermum	Linkage map, ascochyta blight resistance	RAPD, ISSR, STMS, RGA	Collard et al. (2003b)
C. pinnatifidum	Dehydrin gene, drought resistance	cDNA	Bhattarai and Fettig (2005)
C. microphyllum	Metallothionein-like gene	cDNA, rtPCR	Singh et al. (2011)

6.9 Genetic Transformation

Several efforts have also been made for introgression of alien genes from other sources through genetic transformation in order to develop resistance against serious insect pests of chickpea. Sarmah et al. (2004) found that transgenic chickpeas

containing *α-AI1* strongly inhibited the development of *Callosobruchus maculatus* and *C. chinensis* in insect bioassays. Sanyal et al. (2005) successfully expressed the truncated native *Bt cry1Ac* gene in chickpea which showed an effective resistance in transgenic plants against the major pod borer insect, *Helicoverpa armigera*. Similarly, a significant reduction was observed in the survival rate of bruchid weevil *C. maculatus* reared on transgenic chickpea seeds having α-amylase inhibitor gene in a bioassay study (Ignacimuthu and Prakash 2006). In another study, modified *cry2Aa* gene expressed in chickpea was found effective against *pod borer* larva (Acharjee et al. 2010). The genes introgressed through genetic transformation from other than plant sources can flow from transgenic plants to wild relatives, which can cause environmental gene pollution when transgenics are grown in the vicinity of wild relatives (Toker et al. 2006). The genes isolated from wild *Cicer* species belonging to secondary and tertiary gene pools can be more useful in short-term breeding programs using *cis*-genesis approaches.

6.10 Conclusions and Future Prospects

The growth and productivity of the cultivated chickpea is often limited owing to various abiotic and biotic stresses. Unlike the cultivated chickpea, wild *Cicer* species are resistant to multiple abiotic and biotic stresses since selection process for these characteristics during domestication have resulted in narrowing down of the genetic variation of the cultivated chickpea. Lack of adequate genetic variation in the cultivated chickpea can be countered with alien introgressions from wild genetic resources. For this collection, evaluation, documentation, and hybridization of wild *Cicer* species need to be taken on top priority for their efficient utilization in breeding programs. Collection, documentation, and evaluation of some endemic wild *Cicer* species already have top priority because they are endangered in their habitats. A number of studies have clearly shown that transfer of the genes for resistance to abiotic and biotic stresses, and desirable characteristics with yield gene(s) from wild annual species to the cultivated chickpea is possible. Although the transfer of genes from perennial wild *Cicer* species or members of secondary gene pool to the cultivated chickpea has mostly been a failure, some valuable genes have been transferred from the primary gene pool to the cultivated chickpea or incorporated into some breeding lines. Mutation breeding can be strongly advised to increase genetic variation in wild species. Molecular markers that offer a great opportunity have been successfully used for construction of genetic maps of chickpea. Using genetic map of chickpea, identification of QTLs related with quality characteristics and resistance to abiotic and biotic stresses will be useful in breeding programs. Pyramiding of the genes or QTL for development of an ideotype of chickpea can be effectively and cost-effectively monitored with the use of molecular markers. While novel biotechnological approaches will always be in demand for introgression of useful genes from the wild *Cicer* species, approaches such as cisgenesis,

marker assisted breeding and AB-QTL strategy will be required as alternatives and complimentary practices owing to their advantages in precise and target oriented alien gene transfer in chickpea.

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Chapter 7
Pigeonpea

Nalini Mallikarjuna

Abstract Pigeonpea (*Cajanus cajan* L.) is the sixth most important grain legume in the world and second most important pulse crop after chickpea in India. It is a major source of protein for several resource poor rural and urban families of Asia, Africa, the Caribbean, and Latin America and can be cultivated successfully under limited inputs as well as rainfed conditions. Although enormous breeding efforts have been put to the improvement of pigeonpea during the last few decades, the progress in terms of increasing production and quality improvement has not been up to the desired level. One of the major reasons behind this is susceptibility of this crop to many biotic and abiotic stresses, as well as a narrow genetic base of cultivated germplasm thereby offering limited chances of genetic recombination and consequently slower rates of genetic improvement. On the other hand, wild relatives of pigeonpea have evolved naturally to survive environmental extremities including droughts, floods, excess heat, and cold and also have the capability to withstand damage by insect pests and diseases. Owing to these properties, these species have invoked interest of breeders in utilizing them for the improvement of their cultivated counterparts. Consequently, over the past many years, tremendous improvements of practical importance have taken place in pigeonpea utilizing wild genetic resources. This chapter summarizes such significant achievements and highlights the utilization status of wild species in genetic improvement of pigeonpea.

Keywords AB-QTL • Alien gene transfer • *C. platycarpus* • *C. scarabaeoides* • Distant hybridization • Gene pool • Pigeonpea

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7.1 Introduction

In a time of resource constraints, land pressure, environmental concerns, and population explosion, genetic improvement of crops becomes the most promising approach by which food production can meet the demand. For genetic improvement to succeed, plant breeders largely rely on available genetic variability on which they can make selection of desired plant types. Although there is no shortage of genetic variation in nature, a process of genetic erosion due to various reasons, including directed plant breeding, threatens modern varieties. All crop species were originally domesticated from wild plants by humans; the very process of domestication inherently reduced genetic variation (Ladizinsky 1985). The limited genetic variation among modern crop varieties not only makes them more vulnerable to disease and pest epidemics, but it also reduces the chance for plant breeders to identify new and useful combinations of genes, thus causing a slower rate of crop improvement in the long term.

There is renewed interest in the utilization of wild relatives for the improvement of crop plants worldwide. Wild ancestors of most crop plants can still be found in their natural habitats, but their numbers are slowly dwindling. There is an urgent need to conserve all the available germplasm under long-term storage. Germplasm of the crop plants represents a precious source of genetic variation that can potentially insure more rapid and sustained crop plant improvement for many years to come (Tanksley and McCouch 1997).

Pigeonpea [*Cajanus cajan* (L.) Millspaugh] is an important food legume providing protein nitrogen in the human diet to a large population of Asia, Africa, the Caribbean, and Latin America. It has a special place in the diet of vast majority of the Indians who consume pigeonpea in one form or the other in their daily cuisine. It also has wide applications in traditional medicine (van der Maesen 2006). Cultivation of the pigeonpea dates back to at least 3,500 years. The center of origin is the eastern part of peninsular India, including the state of Odisha, where the closest wild relatives (*Cajanus cajanifolia*) occur in tropical deciduous woodlands (van der Maesen 1995). Archaeological finds of pigeonpea include those from two Neolithic sites in Odisha, Gopalpur, and Golabai Sasan dating between 3,400 and 3,000 years ago and sites in South India, Sanganakallu, and Tuljapur Garhi, also dating back to 3,400 years ago. From India it traveled to East Africa and West Africa (Fuller and Harvey 2006). There, it was first encountered by Europeans, so it obtained the name Congo pea. By means of the slave trade, it came to the American continent, probably in the seventeenth century.

Nutritionally, pigeonpea contains high levels of protein and the important amino acids, namely, methionine, lysine, and tryptophan (Saxena et al. 2010). In combination with cereals, pigeonpea makes a well-balanced human diet. Pigeonpea is mostly grown in low-input and risk-prone marginal environments; therefore, there is a large gap between potential yield (2,500 Kg/ha) and actual yields on farmer's fields being 866.2 kg/ha in Asia and 736.2 kg/ha in Africa (Mula and Saxena 2010). Nevertheless, it is a drought-tolerant and a hardy crop, assuring sustainable returns from marginal lands with minimal inputs, hence is considered as a very suitable crop for subsistence agriculture.

Pigeonpea is a cross-pollinated (20–70 %) species with a diploid number of $2n=2x=22$ and genome size of 858 Mbp. Among seed legumes, it is the first plant to have its genome sequenced. The first draft was done by a group of more than 30 scientists from ICRISAT and other institutions as well as ICAR (Singh et al. 2012).

Ample morphological diversity is exhibited by pigeonpea as a crop; however, the same is not true at the molecular level (Yang et al. 2006; Odeny et al. 2007). Low level of genetic diversity has led to a narrow genetic base, and this is reflected by the absence of resistance in this crop to various biotic and abiotic constraints. One of the reasons for low level of genetic diversity is due to the evolutionary bottleneck. There is some evidence that pigeonpea has a monophyletic evolution and has evolved from wild relative *Cajanus cajanifolius* (Kassa et al. 2012). There are 5–6 traits that differentiate *C. cajanifolius* from pigeonpea, such as flower morphology, pod color and morphology, pod constriction, seed color and strophiole, and 100-seed weight (Mallikarjuna et al. 2012a). Molecular studies revealed that a genetic dissimilarity index value ranging from 0.81 to 0.94 exists between the two species (Mallikarjuna et al. 2012a). But the crop has a rich source of variability in the form of wild relatives in different gene pools, which have played a major role in the introduction of disease resistance, good agronomic traits such as high protein content, identification and diversification of cytoplasmic base of CMS system, and more recently introgression of resistance to pod borer (*Helicoverpa armigera*), pod fly, and bruchid (Mallikarjuna et al. 2011a). The classification of Harlan and de Wet (1971) of grouping the germplasm of a crop is followed to group the genetic resources of pigeonpea including their wild relatives. They constituted three basic gene pools and divided them as primary gene pool (GP1), secondary gene pool (GP2), and tertiary gene pool (GP3) and the related genera in the quaternary gene pool (GP4) (Table 7.1, Fig. 7.1).

Table 7.1 Different gene pools of pigeonpea

Primary gene pool	Secondary gene pool	Tertiary gene pool	References
Cajanus cajan, *C. cajanifolius*	*C. acutifolius, C. albicans, C. confertiflorus, C. lanceolatus, C. latisepalous, C. lineatus, C. reticulatus, C. scarabaeoides, C. sericeus, C. trinervius*	*C. goensis, C. heynei, C. kerstingii, C. mollis, C. platycarpus, C. rugosus, C. volubilis*, and other species	Smartt (1990), Singh et al. (2006), Kumar et al. (2011)

Fig. 7.1 Some wild relatives of pigeonpea. (**a**) *C. scarabaeoides*, (**b**) *C. lineatus,* (**c**) *Rhynchosia* spp.

In the genus *Cajanus* with 32 species and 11 related genera, *Cajanus cajan* is the only species cultivated throughout Asia and Africa for its protein-rich grains. The gene bank at ICRISAT conserves over 13,632 accessions of *Cajanus* species from 74 countries. This includes 555 accessions of wild relatives representing 6 genera and 57 species (Upadhyaya et al. 2007). Pigeonpea (*C. cajan* L.) belongs to the subtribe *Cajaninae* and contains 13 genera. Earlier the genera *Atylosia* and *Cajanus* were considered closely related, and later genus *Atylosia* was merged with the genus *Cajanus* (van der Maesen 1980). Subsequently, the genus *Cajanus* has 32 species, 18 of which are endemic to Asia and 13 to Australia and 1 to West Africa (van der Maesen 1986). Apart from these, there are other related genera, namely, *Rhynchosia*, *Dunbaria*, *Flemingia*, *Paracalyx*, *Eriosema*, *Adenodolichos*, *Bolusafra*, *Carissoa*, *Chrysoscias*, and *Baukea*. For details of the gene pools, please refer to the publication of Mallikarjuna et al. (2011a, b).

7.2 Primary Gene Pool

Considerable progress has been made in pigeonpea improvement by using variability within the cultivated species, and consequently, pigeonpea is grown on 4.5 million ha globally with a production of 3.5 million metric tons and productivity of 863 kg/ha (FAO 2009). The gene bank at ICRISAT, Patancheru, India, conserves 13,632 accessions of pigeonpea from 74 countries. This is the single largest collection of pigeonpea germplasm assembled in the world. The germplasm collection includes 8,215 landraces, 4,795 breeding lines, and 67 improved cultivars (Upadhyaya et al. 2012). In spite of the large germplasm collection in primary gene pool, it is not widely used (Wright 1997) as information on the presence of useful traits is not easily available and extended period of research whenever utilized (Goodman 1990). To overcome these issues, core and mini core collections have been developed (Upadhyaya et al. 2006). Variation within the primary gene pool is of utmost importance as accessions belonging to primary gene pool are easy to use with quicker gains and can be directly released as cultivars. Progress has been made in the utilization of material from primary gene pool (Saxena 2000). Pigeonpea varieties BDN-1 and Maruthi released in 1989 are selections from pure line breeding which are popular even today (Bantilan and Joshi 1996). Development of high-yielding varieties such as ICPL 87, ICPL 151, Prabhat, T 21, Pusa Ageti, CO 5, and JA 3 has also been reported (Singh et al. 2005).

In spite of the above successes, a perusal of utilization pattern of *Cajanus* germplasm indicates that so far a very small proportion of germplasm has been used in pigeonpea improvement programs globally. In pigeonpea, 57 ancestors were used to develop 47 varieties. The top 10 ancestors contributed 48 % to the genetic base of the released varieties (Kumar et al. 2003). One of the reasons for such poor utilization may be the vast number of lines available in the primary gene pool which lack characterization, evaluation, and genetic diversity data.

7.3 Secondary Gene Pool

The pigeonpea wild relatives' collection at ICRISAT gene bank has not been characterized and evaluated systematically, although the data is beginning to emerge (Upadhyaya et al. 2012). Main reasons could be low seed quantity, lack of resources, difficulties in phenology and growth habit, and lower priority than the cultivated species (Upadhyaya et al. 2012). However, limited evaluation of different species by researchers across the world indicated that the wild gene pool of pigeonpea, particularly the secondary gene pool, is a promising source for various biotic and abiotic stresses (Bohra et al. 2011; Jadhav et al. 2012).

Compatible wild relatives of pigeonpea which are placed in the secondary gene pool do not need specialized techniques in the crossability experiments in majority of the cases with a few exceptions (Mallikarjuna et al. 2011b). Cytoplasmic male sterility (CMS) systems were developed for pigeonpea exploiting the cross-pollination mechanism and utilizing wild *Cajanus* species. So far eight CMS systems have been reported utilizing wild relatives of pigeonpea (Mallikarjuna et al. 2012b). Of these, seven have been developed utilizing wild relatives from secondary gene pool. One system has cultivated pigeonpea cytoplasm (Mallikarjuna and Saxena 2005). More recently, CMS trait has been observed utilizing *C. lanceolatus*, which is a wild relative in the secondary gene pool (Sandhya and Mallikarjuna, unpublished data). Efforts are underway to identify their restorers. Once developed, this will be named as A_9 CMS system.

The process of outcrossing is important in the development of CMS systems in pigeonpea, but this can lead to genetic deterioration. A partially cleistogamous line, which showed less than 1 % cross-pollination, was purified from the cross *C. cajan* × *C. lineatus*, which was governed by a single recessive gene (Saxena et al. 1992). Partial cleistogamous lines developed from the above cross were found to be stable in India as well as in Sri Lanka. Cleistogamous trait can be utilized in pigeonpea to obtain pure seeds from genetic stocks.

High-protein breeding lines were developed from *C. sericeus*, *C. albicans*, and *C. scarabaeoides*. Significant positive correlation between seed size and protein content was observed in lines derived from *C. scarabaeoides*. Lines HPL 2, HPL 7, HPL 40, and HPL 51 are some of the high-protein and high-seed-weight lines derived from wild species (Saxena et al. 1987). More recently crosses between pigeonpea and *C. acutifolius* yielded progeny with high seed weight. High seed weight accompanied by beige seed color is a desirable trait (Jadhav et al. 2012).

C. acutifolius, a wild relative from secondary gene pool and native of Australia, can be crossed with pigeonpea as a one-way cross. The reciprocal cross using *C. acutifolius* as the female parent aborts to give rise to immature seeds. In vitro interventions are necessary to obtain hybrid plants (Mallikarjuna and Saxena 2002). Advanced generation population from the cross utilizing *C. acutifolius* as the pollen parent has shown resistance for pod borer damage (Mallikarjuna et al. 2007), variation for seed color, and high seed weight. Some of the lines showed high level of resistance to pod borers, pod fly, and bruchid under unprotected field conditions

(Jadhav et al. 2012). Bruchid resistance (Jadhav et al. 2012) is an important trait for pigeonpea seeds under storage as resistance to the pest has not been observed in cultivated pigeonpea. These lines are available in pigeonpea breeding and Legume Cell Biology Units of Grain Legumes Program (CGIAR project on Grain Legumes).

Some of the advance generation lines derived from *C. acutifolius* were screened for waterlogging by germinating them and later growing them under waterlogged conditions. A few lines grew under waterlogged conditions, and formation of lenticels was observed in the region above the water surface. The region gave rise to roots which entered the soil through the water surface. This shows that some of these lines that may survive were resistant to waterlogged conditions (Aneesha Begum and Nalini Mallikarjuna, unpublished data).

Another species from secondary gene pool, namely, *C. lanceolatus*, was crossed successfully with cultivated pigeonpea at ICRISAT and progeny lines developed. F_1 hybrids flowered, but some of the hybrids were pollen sterile, and in the rest of the hybrids, pollen fertility varied from 25 to 55 %. All the hybrids were female fertile. Progeny lines developed from the cross were screened for bruchid resistance. *C. lanceolatus* inhibited bruchid growth and survival. Some of the lines showed delayed bruchid growth and delayed life cycle, thus showing antibiosis mechanism of resistance to bruchids. Lines were screened for protein content, and some of the lines showed higher protein content than both their parents. Further biochemical analysis showed higher content of proteinase inhibitors activity in some of the lines (Sandhya Srikanth and Nalini Mallikarjuna, unpublished data). A new source of CMS was identified in the progeny lines, and experiments are underway to identify maintainers and restorers. Sateeshkumar (1985) attempted crossing pigeonpea with *C. lanceolatus* but obtained hybrids which did not flower and remained in the vegetative stage.

7.4 Tertiary Gene Pool

There are 20 wild species in the tertiary gene pool of pigeonpea (Mallikarjuna et al. 2011a, b,). Until now, only one wild *Cajanus* species from this gene pool was amenable to interspecific hybridization and gene transfer (Mallikarjuna et al. 2011b). *C. platycarpus* was successfully crossed utilizing hormone-aided pollinations and in vitro interventions to obtain hybrids. Progeny lines showed variation for days to flower; growth habit; seed weight and number; seed color; resistance to pod borer, pod fly, and bruchids; and cytoplasmic male sterility. Some chasmogamous lines were identified in CMS lines, a trait favoring total cross-pollination. Hence utilizing *C. platycarpus* not only broadened the genetic base of pigeonpea, but it was possible to introgress useful traits. Diversity Array Technology (DArT), a genome-wide marker technology, was used to genotype the parents and advance generation hybrids after four backcrosses. A total of 1,225 markers were found polymorphic among the parents and the progeny. The results of the study showed that apart from DNA stretches from the female and male parent, there was some novel DNA polymorphism observed in the progeny not seen in both the parental species. It was

interesting to observe that as per theoretical calculations, there should be 3.12 % of *C. platycarpus* genome after four backcrosses with cultivated parent *C. cajan* (Mallikarjuna et al. 2011a). Diversity Array Technology analysis showed the presence of *C. platycarpus* genome ranging from 2.0 to 4.8 %. The presence of non-parental DNA sequences was presumably because of recombination, ranging from 2.6 to 10.4 % (Mallikarjuna et al. 2011a).

More recently another species from tertiary gene pool, namely, *C. volubilis*, was crossed with pigeonpea. In F_2 generation, extra short-duration lines were recovered. These lines flowered earlier than the short-duration cultivar ICPL 85010 which was the female parent of the cross. The lines were again screened for the short-duration trait in rabi 2012 and were found to retain the trait. Dwarf growth habit and determinate and semi-determinate plants were observed. In the determinate types, the number of pods per inflorescence and the number of inflorescence was more than that observed in the extra-early and determinate cultivar MN5 (Sandhya Srikanth and Nalini Mallikarjuna, unpublished data).

7.5 Quaternary Gene Pool

There are 11 related genera, namely, *Rhynchosia*, *Flemingia*, *Dunbaria*, *Erisema*, *Paracalyx*, *Adenodolichos, Bolusafra*, *Carissoa*, *Chrysoscias*, and *Baukea* including *Cajanus* under the subtribe Cajaninae.

Many of these genera are classified as underexploited legumes. *Rhynchosia* is one such example as it harbors important nutritional and therapeutic properties, with the presence of phytochemicals such as alkaloids, glycosides, anthraquinones, carotenoids, coumarins, dihydrochalcones, fatty acids, flavonoids, steroids, and triterpenoids (Bakshu and Venkataraju 2001). Some species of *Rhynchosia* are used as human and animal diet (Oke et al. 1995). Many of the tribal communities in India soak the seeds in water and consume the seeds after boiling and decanting many times (Murthy and Emmannuel 2011). Apart from this, many of the *Rhynchosia* species are known to exhibit antitumor and thus curative properties. Normally during cancer treatment iron deficiency and anemia are major issues. It was observed that treatment with *Rhynchosia* seeds restored hemoglobin (Hb) count and RBC and WBC count to normal levels. With the interest in dietary flavonoids and suppression of cancer, *Rhynchosia* species are surely going to attract more attention in the coming days.

None of the genera in the quaternary gene pool have been successfully crossed with pigeonpea. Among the genera in the quaternary gene pool, *Rhynchosia* was selected to initiate crossing/introgression/gene transfer experiments as it had many desirable properties, as listed above. It was possible to successfully cross *Rhynchosia* with pigeonpea through hormone-aided pollinations. The success rate of crossing *Rhynchosia* was low, not exceeding 1–2 %, but it was possible to obtain hybrids. Screening the hybrids with molecular markers confirmed the hybridity (Nalini Mallikarjuna and Rajeev Varshney, unpublished data). Although the initial processing

of crossing was challenging, nevertheless, hybrids were obtained. They were fertile and it was possible to obtain self and backcross progenies. Experiments to screen and study the progeny lines for different traits/constraints are in progress.

7.6 AB-QTL Mapping Populations Utilizing Wild *Cajanus* Species

Advance backcross quantitative trait loci detection method abbreviated as AB-QTL increases the efficiency of identifying and transferring beneficial alleles from exotic germplasm (Tanksley and Nelson 1996). This method instead of using traditional F_2 or RIL mapping populations, involves two or three backcrosses to the recurrent parent during population development, thus reducing the amount of donor introgressions in each individual. This method is especially advantageous where wild relatives are used to develop mapping populations. AB-QTL method is used for simultaneous discovery and transfer of valuable QTLs from wild relatives. Since QTL analysis is delayed till BC_2 or BC_3 generations, it may be possible to detect dominant, partially dominant, over dominant, additive, and epistatic QTLs to name a few.

Introgression of useful genes/traits accompanied by undesirable genomic fractions harboring deleterious alleles, collectively called linkage drag, can be overcome to identify favorable exotic quantitative trait locus (QTL) alleles for the improvement of agronomic traits. Two wild relatives, namely, *C. cajanifolius* (the progenitor species) and *C. acutifolius*, a wild species from the secondary gene pool and with many desirable traits (Mallikarjuna et al. 2011a), were used to develop AB-QTL mapping populations. The populations are ready for phenotyping and genotyping after two backcrosses and selfings. It is envisaged that such mapping populations will identify useful alleles present in the wild species as observed in other crops such as rice (Septiningsih et al. 2003).

7.7 Conclusions

No other leguminous crop has been investigated for alien introgression and succeeded in crossing wild relatives form all the gene pools, namely, secondary, tertiary, and quaternary gene pools. Pigeonpea is one crop where tremendous progress has been made to cross wild *Cajanus* species from different gene pools and introgress genes/traits successfully. With these successes it can no more have a narrow genetic base. With the advances in pigeonpea genomics, and a major effort in sequencing the crop, and success in wide crosses in pigeonpea, it has emerged from being labeled as an orphan crop to a trend setter. Recent successes in wide crosses show that it is possible to introduce desirable traits such as pod borer resistance, develop CMS systems, develop lines with multiple disease and pest resistance, change plant type, and increase seed weight and yield.

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Chapter 8
Vigna

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Abstract The genus *Vigna* comprises more than 200 species of which 7 are of tremendous agronomic importance. These are grown mainly in the warm temperate and tropical regions of the world. Valued for their grains with high and easily digestible proteins, these crops are also known as forage, green manure, and cover crops. Due to a short life cycle, these are suitable as catch crops and also fit well in intercropping, mixed or relay cropping. For genetic improvement of cultivated vignas, mainly cultivated germplasm and exotic lines have been used so far. However, despite development of several improved cultivars in different *Vigna* crops, biotic and abiotic stresses still remain the major constraints in realizing their true yield potential. While plethora of genes conferring resistance/tolerance to these stresses have already been transferred from cultivated germplasm, wild genetic resources offer additional sources of useful alien variation which can be incorporated in cultivated *Vigna* through alien gene transfer. With better understanding of the processes behind pollen germination and pollen tube growth, fertilization, embryo and endosperm development and inheritance pattern, strategies have been developed to avoid pre- and post-fertilization barriers in successful distant hybridization leading to alien gene transfer. These include making reciprocal crosses, repeated pollination, hormonal treatment of flower buds, polyploidization, use of bridge species and most importantly, embryo rescue which have increased success rate of alien gene transfer in *Vigna* through sexual hybridization. Nevertheless, alien gene transfer through genetic transformation and use of molecular breeding tools still lag behind in *Vigna* and therefore need special attention. The significant achievements made in different *Vigna* species in alien gene transfer and their utilization have been discussed in this chapter.

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8.1 Introduction

The genus *Vigna* is a large and immensely variable genus comprising more than 200 species. This genus contains several agronomically important species that have considerable economic and environmental importance. These include the mung bean [*V. radiata* (L.) Wilczek], urd bean [*V. mungo* (L.) Hepper], cowpea [*V. unguiculata* (L.) Walp], azuki bean [*V. angularis* (Willd.) Ohwi & Ohashi], bambara groundnut [*V. subterranea* (L.) Verdn.], moth bean [*V. aconitifolia* (Jacq.)], and rice bean [*V. umbellata* (Thunb.) Ohwi & Ohashi]. Most of these vignas are grown in the warm temperate and tropical regions of the world. The major areas producing these crops include countries from Asia, Australia, West Indies, South and North America, and tropical and subtropical Africa. Cultivated vignas are important to human nutrition in a large part in tropical Asia and also Africa in the predominantly vegetarian diets. Many of the above species besides their high protein grains are valued as forage, green manure, and cover crops.

While the production statistics are not available for individual *Vigna* species, the economically important *Vigna* crops are grouped under the dry beans, and the annual worldwide production of the various *Vigna* species approaches to about 23.29 million tonnes from an area of 28.98 million hectares (FAOSTAT 2013). *Vigna* species have a number of attributes that make them valuable in cereal-based cropping systems as well as a suitable candidate for relay cropping and intercropping. Their suitability to extreme environments, wider adaptability, a comparatively shorter life cycle, low input requirements, and nitrogen-fixing ability increases their importance in subsistence farming which is a typical characteristic of agriculture in the developing world (Pratap et al. 2013a). These are well suited to a large number of cropping systems and constitute an important place in vegetarian diets. The nutritional value of these crops is exceptionally high, and the germinated seeds have nutritional value compared with asparagus or mushroom. The germinated seeds (i.e., sprouts) of mung bean have good nutritional value as with sprouting, there is an increase in the thiamine, niacin, and ascorbic acid concentration. The seeds contain approximately 25–28 % protein, 1.0–1.5 % oil, 3.5–4.5 % fiber, 4.5–5.5 % ash, and 62–65 % carbohydrates on dry weight basis. Owing to above properties, there is an ample scope of a vertical as well as horizontal expansion of these crops in the world. Furthermore, being the crops of mainly the Asian and African continent and not being a mandate of international institutes like ICRISAT and ICARDA, these crops are still to get their due share in research and development and hence offer great opportunities to work with.

Development of disease- and insect-pest-resistant, short-duration, and photo-thermo insensitive varieties during the last three decades has helped in expanding their area.

A relatively recent approach of researchers towards introgression of useful genes from wild into the cultivated background through distant hybridization as well as through genetic transformation is further expected to develop more promising material in the years to come. Wild and exotic germplasm offers new sources of variability hitherto not found in the cultivated species and therefore provides additional avenues of selection for agronomic traits. Several new recombinants with desirable agronomic traits and cultivars with improved characteristics have already been developed through wide crosses, while a few cultivars have also been developed in crops like mung bean and urd bean. Noteworthy progress has also been in crops like cowpea using genetic transformation. This chapter elaborates such novel efforts and provides an insight into the successes and failures of alien gene transfer for improvement of major *Vigna* crops.

8.2 Origin, History, and Evolution

The origin of a species is generally determined on the basis of information available on archeological remains as well as existence of wild species or the progenitors. The natural forces including mutation, migration, hybridization, and genetic drift made alteration in the wild species resulting in evolution of the wild ancestors of cultivated species (Pratap and Kumar 2011). Mung bean (*Vigna radiata* var. *radiata*) and urd bean (*V. mungo*), the most important *Vigna* crops, are believed to have originated in the Indian subcontinent (deCandolle 1884; Vavilov 1926; Zukovskij 1962). Since India has a wide range of genetic diversity of cultivated as well as of weedy wild types of mung bean, it is considered as the region of its first domestication (Baudoin and Marechal 1988). The progenitors of mung bean, *V. radiata* var. *sublobata*, and urd bean, *V. mungo* var. *silvestris*, are seen in abundance as weeds in cultivated and wasteland areas of India (Singh et al. 1974; Chandel et al. 1984; Lawn and Cottell 1988) as well as in wetlands in subtropical regions of northern and eastern Australia (Lawn and Cottell 1988). Considerable amount of genetic variability have been observed to be found in the Western *Ghats* on roadsides, grasslands, and uninhabited areas (Aditya Pratap and Joseph K. John, unpublished data). The origin and domestication of cowpea (*V. unguiculata*) is believed to have occurred in the West or Central Africa, where plenty of wild and weedy types are found in the forests and Savannah (Harlan 1971; Rawal 1975). The archeological evidences from West Africa date back the existence of cowpea to 3000 BC. It is likely that this crop was first introduced to India during the Neolithic period, and therefore, India appears to be a secondary center of origin of this crop. Vavilov (1928, 1949), nevertheless, recognized India as the main center of origin of this crop, while Africa and China were considered as the secondary centers.

For rice bean (*V. umbellata*), Vavilov (1926) designated India as the center of origin for its cultivated and wild forms, inclusive of Assam and Burma but exclusive of northwest India. The wild form var. *gracilis* is likely to be an ancestor of rice bean.

Other wild vignas, viz., *V. minima* (Roxb.) Ohwi & Ohashi and *V. delzelliana*, seem to have much similarities with var. *gracilis* (Marechal et al. 1978). *V. minima* was considered as a wild relative of rice bean which has been found located in Western Ghats and Kerala (Gopinathan and Babu 1986).

Moth bean (*V. aconitifolia*) is considered as one of the most primitive *Vigna* crops in respect of its evolution (Smartt 1985). Vavilov (1926) mentioned India as its center of origin, while Marechal et al. (1978) opined that Sri Lanka and Pakistan are the centers of diversity of this crop. Similarly, azuki bean (*V. angularis*) originated in the Chinese center (Vavilov 1926), and the *V. angularis* var. *nipponensis* (Yamaguchi 1992; Kaga et al. 2008) is presumed as the wild ancestor of cultivated azuki bean. It exists as a crop complex in Japan where cultivated, wild, and weedy azuki beans are found (Vaughan et al. 2005).

8.3 The Gene Pool Concept

Grouping of species in different gene pools helps breeders decide which species is to be used in hybridization programme for obtaining hybrid seeds and subsequently viable progenies from the crosses. The gene pool concept of Harlan and de Wet (1971) has been tremendously useful to plant breeders for initiating a pre-breeding program for directed crop improvement (Kumar et al. 2011). Various *Vigna* species have been grouped into primary, secondary, and tertiary gene pools on the basis of crossability and cytogenetic, phylogenetic, and molecular data. Table 8.1 elaborates the species in the secondary and tertiary gene pools on the basis of *V. radiata* and *V. mungo* put in primary gene pool. Among the wild species, occurrence of useful

Table 8.1 Different gene pools of mung bean and urd bean

Crop	Primary gene pool	Secondary gene pool	Tertiary gene pool	References
Mung bean	*Vigna radiata* var. *radiata*, *V. radiata* var. *sublobata*, *V. radiata* var. *setulosa*	*V. mungo var. mungo*, *V. mungo* var. var. *silvestris*, *V. aconitifolia*, *V. trilobata*	*V. angularis*, *V dalzelliana*, *V. glabrescens*, *V. grandis*, *V. umbellata*, *V. vexillata*	Chandel and Laster (1991); Dana and Karmakar (1990); Smartt (1981, 1985); Kumar et al. (2004)
Urd bean	*V. mungo* var. *mungo*, *V. mungo* var. *silvestris*	*Vigna radiata* var. *radiata*, *V. radiata* var. *sublobata*, *V. radiata* var. *setulosa*, *V. aconitifolia*, *V. trilobata*	*V. angularis*, *V. dalzelliana*, *V. glabrescens*, *V. grandis*, *V. umbellata*, *V. vexillata*	Chandel and Laster (1991); Dana and Karmakar (1990); Kumar et al.(2004)

Source: Kumar et al. (2011)

genes is much more frequent in the secondary and tertiary gene pools (Mallikarjuna et al. 2006; Tullu et al. 2006). However, successful hybridization between the cultivated vignas and their wild relatives in secondary and tertiary gene pools is constrained by crossability barriers, and therefore, their successful utilization in crop improvement programs requires special efforts and use of novel techniques such as embryo rescue, poplyploidization, reciprocal crossing, hormonal manipulations, and use of bridge species.

8.4 Wild Species: Potential Source of Alien Variation

The success of distant hybridization and subsequent alien gene transfer mostly depends upon the pollination behavior, frequency of pollination, nature and direction of the cross, ploidy status of species involved in the cross, and use of special techniques. Besides the above, the level of already existing variability within a species is also a crucial factor in deciding whether distant hybridization is to be taken up for alien gene transfer because distant hybridization is usually a long and time-consuming process and may sometimes yield no results, frustrating breeders' efforts. Wild relatives of *Vigna* can offer sources for imparting resistance to several biotic and abiotic stresses besides improving yield and quality traits (Pratap et al. 2012a). This can be achieved by utilizing the collected plant genetic resources of *Vigna* through pre-breeding, which will develop more acceptable lines to the plant breeders. Wide diversity was observed in 45 morphological characters for 206 accessions of 14 wild *Vigna* species, viz., *V. khandalensis*, *V. radiata* var. *sublobata*, *V. radiata* var. *setulosa*, *V. mungo* var. *silvestris*, *V. hainiana*, *V. umbellata*, *V. dalzelliana*, *V. bourneae*, *V. minima*, *V. trilobata*, *V. aconitifolia*, *V. vexillata*, *V. pilosa*, and *V. glabrescens* (Bisht et al. 2005). Similarly, wide genetic variability for 37 morphological traits has been observed in 62 accessions of 18 *Vigna* species including *V. trilobata*, *V. umbellata*, *V. radiata* var. *radiata*, *V. dalzelliana*, *V. mungo* var. *mungo*, *V. hainiana*, *V. radiata*, *V. pilosa*, *V. vexillata*, *V. mungo* var. *silvestris*, *V. radiata* var. *sublobata*, *V. radiata* var. *setulosa*, *V. mungo*, *V. unguiculata*, *V. trinervia* var. *bournei*, *V. trinervia*, *V. glabrescens*, *and V. mungo* var. *silvestris* (Pratap et al. 2012b, Aditya Pratap, unpublished data, Fig. 8.1a, b). The sub-gene pool of wild types in accession PLN 5 of *V. radiata* var. *sublobata* (Singh and Ahuja 1977) and IW 3390 of *V. mungo* var. *silvestris* (Reddy and Singh 1993) have been identified as potential sources of MYMV resistance, and TC 1966 of *V. radiata* var. *sublobata* was identified to carry bruchid tolerance gene (Tomooka et al. 1992). In cowpea, the most important pests are the post flowering insect-pests including legume pod borers and pod-sucking bugs. Resistance to these sources has been found in *V. vexillata* (Fatokun 1991). Similarly, variation for yield components and Mung bean Yellow Mosaic Virus (MYMV) resistance is available in *V. mungo* var. *silvestris* and a few accessions of the wild progenitor *V. radiata* var. *sublobata* (Singh 1990). A wild accession of

Fig. 8.1 (**a**) Morphophysiological variation in wild accessions of *Vigna* species—A and B: *V. glabrescens* (accession IC251372); C, D & E: *V. umbellata* (accession PRR-2008-2) F, G, and H: *V. radiata* (accession JAM-09-29); I and J: *V. unguiculata*; K and L: *V. trilobata* (accession JAP10-5).

Vigna radiata var. *sublobata*, PLN 15, is found to be the potential donor for pods per plant and seeds per pod (Reddy and Singh 1990). Resistance to MYMV has also been reported in *V. umbellata*, *V. trilobata,* and *V. mungo* (Nagaraj et al. 1981; Singh et al. 2003). *Vigna mungo* var. *silvestris* is reported to be immune to bruchids (Fujii et al. 1989; Dongre et al. 1996). Recently, rice bean (*V. umbellata*) is identified as most useful because many accessions show complete resistance or immunity to the bruchids and it is a cultivated species; therefore, gene transfer from rice bean into mung bean and urd bean may be comparatively easier. Efforts are in progress at AVRDC, Taiwan and IIPR, India to utilize *V. radiata* var. *sublobata* for resistance to bruchids. Table 8.2 summarizes the wild *Vigna* resources which may be used as potential donors for various traits.

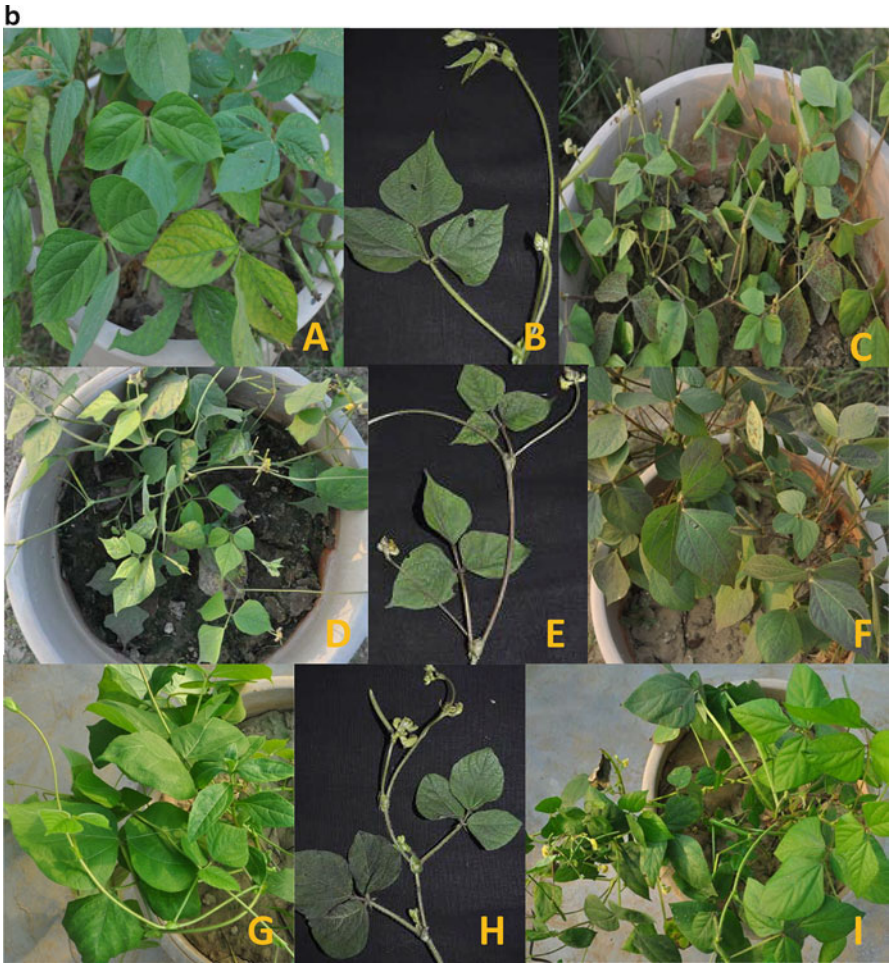

Fig. 8.1 (continued) (**b**) Morphophysiological variation in wild accessions of *Vigna* species—A and B: *V. trinervia* (JAP10-51) C: *V. radiata* var. *sublobata* (IC 251416); D and E: *V. hainiana* (IC 331450); F: *V. mungo* var. *mungo* (IC 251386); G and H: *V. radiata* var. *setulosa* (IC 251423); I: *V. radiata* var. *radiata* (IC 251425)

8.5 Crossability Studies

Successful crossing between the crop cultivars and wild species is a prerequisite for alien gene introgression through sexual hybridization. However, many of the wild species are not crossable with their cultivated counterparts due to pre- and post-fertilization barriers and consequently are of no use for crop improvement. Besides genetic factors, environmental factors can also influence embryo development of interspecific hybrids and thereby the crossability potential (Percy 1986; Sirkka et al. 1993; Tyagi and Chawla 1999). Extensive efforts have been put for the development

Table 8.2 Potential sources of alien variation in *Vigna*

Character	Species	References
Resistance to bruchid	*V. riukinensis*	Tomooka et al.(1992)
	V. reflexo-pilosa	Tomooka et al. (1992)
	V. radiata var. *sublobata*	Fujii and Miyazaki 1987; Kaga and Ishimoto (1998); Miyagi et al. (2004)
	V. umbellata	Tomooka et al. (2000); Kashiwaba et al. (2003); Somta et al. (2006)
	V. tenuicaulis	Tomooka et al. (2000)
	V. nepalensis	Somta et al. (2008a)
Resistance to cowpea storage weevil	*V. vexillata*	
	V. reticulata	
	V. oblongifolia	
	V. luteola	
Resistance to powdery mildew	*V. stipulaceae*	Tomooka et al. (2006a)
	V. reflexo-pilosa var. *glabra*	Egawa et al. (1996)
Low trypsin inhibitor activity	*V. tenuicaulis*	Konarev et al. (2002)
Chymotrypsin absent	*V. grandiflora*	Konarev et al. (2002)
High methionine content	*V. radiata* var. *sublobata*	Babu et al. (1988)
High photosynthetic efficiency and drought tolerance	*V. radiata* var. *sublobata*	Ignacimuthu and Babu (1987)
Drought tolerance	*V. aconitifolia*	Jain and Mehra (1980)
Heat tolerance	*V. aconitifolia*	Tomooka et al. (2001)
	V. riukinensis	Egawa et al. (1999)
Insect resistance	*V. unguiculata* ssp. *dekindtiana* var. *pubescens*	Ehlers and Hall (1997)
Antibiosis to cowpea moth	*V. unguiculata* ssp. *mensensis*	Ezuch (1982)
YMV resistance	*V. radiata* var. *sublobata*	Singh and Ahuja (1977)
Cucumber mosaic virus resistance	*V. reflexo-pilosa* var. *glabrescens*	Egawa et al. (1996)
Bean fly resistance	*V. reflexo-pilosa*	Egawa et al.(1996)
High tolerance to saline and Alkaline soils	*V. radiata* var. *sublobata*	Lawn et al. (1988)
Resistance to pod bug	*V. unguiculata* ssp. *dekindtiana* TVNu 151	Koona et al. (2002)
Resistance to cowpea insects pests	*V. vexillata*	Birch et al. (1986), IITA (1988)
No. of seeds/plant and pods/plant	*V. radiata* var. *sublobata*	Reddy and Singh (1990)
Resistance to yellow mosaic virus	*V. radiata* var. *sublobata*	Reddy and Singh (1990), Pal et al. (2000)
	V. trilobata	Nagaraj et al. (1981)
	V. umbellata, *V. trilobata*, *V. mungo*	Pandiyan et al. (2008)
Photo-thermo insensitivity	*V. umbellata*, *V. glabrescens*	Pratap et al. (2012b)

Table 8.3 Crossability studies in *Vigna* species

Name of the cross	Reference
V. mungo × *V. trilobata*	Dana (1966)
V. mungo × *V. umbellata*	Ahn and Hartmann (1978b); Biswas and Dana (1975)
V. mungo × *V. mungo* var. *silvestris*	Reddy and Singh (1989)
V. mungo × *V. angularis*	Chen et al. (1983); Ahn and Hartmann 1978a, 1978b
V. mungo × *V. dalzelliana*	Chavan et al. (1966)
V. mungo × *V. glabrescens*	Dana (1968); Krishnan and De (1968)
V. mungo × *V. radiata*	Gosal and Bajaj (1983a, b); Verma and Singh (1986)
V. radiata × *V. mungo*	Chavan et al. (1966); Dana (1966); De and Krishnan (1966); Subramanian (1980); Miyazaki (1982); Chen et al. (1983); Gill et al. (1983); Shanmungam et al. (1983); Verma and Singh (1986); Ahn and Hartmann (1978a, b); Singh et al. (1996); Subramanian and Muthiah (2001); Singh and Dikshit (2002)
V. radiata × *V. angularis*	Sawa (1973); Ahn and Hartmann (1978a, b); Chen et al. (1983)
V. radiata × *V. glabrescens*	Krishnan and De (1968); Chen et al. (1989)
V. radiata × *V. grandis*	Chavan et al. (1966)
V. radiata × *V. mungo* var. *silvestris*	Singh and Ahuja (1977); Miyazaki (1982)
V. radiata × *V. trilobata*	Chavan et al. (1966); Dana (1966); Bharathi et al. (2006); Pandiyan et al. (2012)
V. radiata × *V. umbellata*	Dana (1966); Sawa (1973); Machado et al. (1982); Pandiyan et al. (2008); Bharathi et al. (2006)
V. radiata × *V. radiata* var. *sublobata*	Pandiyan et al. (2010)
V. radiata var. *setulosa* × *V. mungo*	Karmakar and Dana (1987)
V. radiata var. *sublobata* × *V. mungo*	Biswas and Dana (1975); Miyazaki (1982); Miyazaki et al. (1984)
V. radiata var. *sublobata* × *V. mungo* var. *silvestris*	Miyazaki (1982); Miyazaki et al. (1984)
V. radiata × *V. dalzelliana*	Pandiyan et al. (2010)
V. radiata × *V. aconitifolia*	Bharathi et al. (2006)
V. unguiculata × *V. unguiculata* var. *pubescens*	Mohammed et al. (2010)
V. unguiculata × *V. vexillata*	Barone et al. (1992)
V. pubescens × *V. unguiculata*	Fatokun and Singh (1987)

of interspecific crosses in *Vigna* in the last two decades, and consequently, a plethora of information has been generated relating to gene flow between cultivated *Vigna* species and their wild relatives, crossability barriers and methods to overcome them, thereby enhancing the interest of breeders in distant hybridization (Table 8.3). The early work on interspecific hybridization in *Vigna* has been reviewed by several workers (Singh 1990; Singh et al. 2003).

Most of the reports studying crossability among different *Vigna* species suggest that *V. radiata* produced successful hybrids as a female parent with *V. mungo*,

V. umbellata, and *V. angularis*, while their reciprocal crossing was not successful. Nevertheless, by using sequential embryo rescue, the reciprocal hybrids between *V. mungo* and *V. radiata* could be successfully obtained (Gosal and Bajaj 1983a; Verma and Singh 1986). *V. mungo* has also been reported to cross successfully with *V. glabrescens* (Dana 1968; Krishnan and De 1968), *V. trilobata* (Dana 1966), and *V. dalzelliana* (Chavan et al. 1966). Similarly, *V. radiata* × *V. umbellata* crosses were generated to transfer resistance to MYMV and other desirable traits into mung bean (Verma and Brar1996). More recently, Pal et al. (2005) successfully produced interspecific crosses between *V. mungo* and *V. umbellata*.

Mung bean × rice bean crosses were generated to incorporate MYMV resistance and other desirable traits into mung bean (Verma and Brar 1996). However, genotypic differences contributed in success of the cross. Four amphidiploids of mung bean (ML 267 and K 851) × rice bean (RBL 33 and RBL 140) crosses were successfully produced and evaluated for different characters (Dar et al. 1991). Singh et al. (2003) also produced successful hybrids between *V. radiata* and *V. umbellata*, and the hybrids possessed intermediate morphology with MYMV resistance. Interspecific hybridizations between cultivated cowpea (*V. unguiculata* ssp. *unguiculata* and *V. unguiculata* ssp. *biflora*) and wild forms of cowpea (*V. unguiculata* var. *spontanea*, *V. unguiculata* ssp. *alba*, *V. unguiculata* ssp. *stenophylla*, *V. unguiculata* ssp. *pawekiae*, and *V. unguiculata* ssp. *baoulensis*) were attempted by Kouadio et al. (2007), and the highest success rate was obtained in crosses between cultivated and annual inbred forms, though hybridization between cultivated and wild allogamous forms gave an intermediate rate of success. The success rate was lower when *V. unguiculata* ssp. *baoulensis* was crossed with cultivated forms.

Sidhu (2003) produced interspecific hybrids of *V. radiata* with *V. mungo* and *V. trilobata*. Though the crosses between *V. radiata* and *V. trilobata* were successful, the seeds produced between *V. mungo* and *V. trilobata* had poor germination, and the germinated seedlings did not survive. The cytological analysis revealed irregular chromosome behavior at diakinesis and/or at metaphase I. In some of the interspecific crosses of *Vigna*, the hybrid sterility has been observed to be of segregational type and was mainly due to interchange, inversion, and possible duplication-deficiency type of structural chromosomal abnormalities in the F_1 individuals (Dana 1964; Biswas and Dana 1975; Karmakar and Dana 1987). Pandiyan et al. (2008) suggested that male sterility in interspecific hybrids was due to meiotic irregularities, viz., unequal separation of tetrads, while the female sterility was due to degeneration of megaspore during megasporogenesis. They attempted direct interspecific crosses in *V. radiata* with two accessions of *V. umbellata*. Despite the crossability barriers being predominant, they were able to obtain interspecific hybrids in these crosses, the phenology of the F_1 plants being intermediate while the reproductive parts resembling those of *V. umbellata*.

Similarly, Bharathi et al. (2006) studied the crossability relationship of seven cultivars of *V. radiata* with six accessions of *V. umbellata*, five of *V. aconitifolia*, and one each of *V. trilobata* and *V. sublobata*. Among these, higher rate of crossability was observed in the cross *V. radiata* × *V. umbellata*. Surprisingly the success rate in the reciprocal cross-combination (*V. umbellata* × *V. radiata*) was very low. In another

study, Mohammed et al. (2010) reported that high level of cross-compatibility existed between the cultivated cowpea (*V. unguiculata*) and its wild relative var. *pubescence*.

8.6 Crossability Barriers

Reproductive isolation developed during the process of speciation, embryo or endosperm abortion, hybrid sterility and limited levels of genetic recombination are the major obstacles for successful alien gene transfer in crop plants through sexual hybridization. Crossability barriers prevent successful hybridization between species of different gene pools and pose a major hindrance to breeders' efforts in alien gene transfer. These barriers can manifest themselves through reduced fertilization, reduction in number of hybrid seeds or development of abnormal—shriveled, small, or non-viable—seeds, and retarded development of hybrid endosperm leading to embryo death or hybrid sterility. Besides these, the undesirable linkages to non-agronomic traits (linkage drag) pose further problems during selection of desirable recombinants once gene flow has been achieved. The nature also supports these barriers to avoid extinction of species by muddled hybridization (Kumar et al. 2011). Like other crops, in *Vigna* also, several crossability barriers have been reported, the most common being cross-incompatibility, embryo abortion at early growth stage, inviability of F_1 hybrids, and sterility of F_1 hybrid and subsequent progenies. The pre-fertilization cross-incompatibility between parent species arises when pollen grains do not germinate or the pollen tube does not reach ovary or the male gametes do not fuse with female gametes (Chowdhury and Chowdhury 1983; Shanmungam et al. 1983).

In *Vigna* crops, slow rate of pollen growth, in addition to abnormalities in stigmatic and stylar regions, has been reported to be the major cause for low percentage of pod set in *V. radiata* × *V. umbellata* and *V. mungo* × *V. umbellata* crosses (Thiyagu et al. 2008). Crossability was reported to be genotype dependent by Rashid et al. (1988), hence leading to cross-incompatibility in some particular combinations, while the other cross-combinations were successful. Kumar et al. (2007) observed that strong pre-fertilization barriers were present in the cross between *V. radiata* and *V. umbellata*, and growth and lethality of interspecific hybrid seedlings were influenced by the genotypes of both the parental species. Meiotic irregularities have also been reported to be a major factor for cross-incompatibility. Sidhu (2003) attempted interspecific hybridization of *V. radiata* with *V. mungo* and *V. trilobata*.

Difference in style length may not be a major crossability barrier in case of *Vigna* species as the long-styled female parent *V. radiata* could be successfully crossed with short-styled male parent *V. trilobata*. Similarly, ploidy level was also not reported to affect crossability as crosses were obtained successfully between diploid × tetraploid (*V. radiata* × *V. glabrescens*) (Chen et al. 1989; Krishnan and De 1968) and tetraploid × diploid (*V. glabrescens* × *V. umbellata*) *Vigna* species. Post-fertilization barriers of varying degrees have also been reported in most of the

interspecific *Vigna* crosses (Dana 1964; Biswas and Dana 1975; Machado et al. 1982; Chen et al. 1983; Gopinathan et al. 1986; Al-Yasiri and Coyne 1966; Bharathi et al. 2006; Pandiyan et al. 2010; Chaisan et al. 2013). These affect the successful recovery of desirable recombinants through development of shriveled hybrid seed with reduced or no germination (hybrid inviability), development of dwarf and non-vigorous plants and death of F_1 plants at critical stages of development (hybrid lethality).

8.7 Strategies to Overcome Crossability Barriers in *Vigna*

Over the last one and a half decade, convincing evidence at both morphological and molecular levels has come forth for utility of wild progenitors and related species as donors of productivity alleles (Kumar et al. 2011). With better understanding of the processes involved in pollen stigma interaction, pollen germination, pollen tube growth, and fertilization, the ability to overcome crossability barriers and produce viable and fertile interspecific hybrids has increased tremendously. Accordingly, a number of procedures have been developed to overcome barriers that hinder the free exchange of genes between distantly related species. These include reciprocal crossing, repeated pollination, hormonal treatment of flower buds prior to or after pollination, use of different accessions of both the species, polyploidization followed by hybridization, polyploidization of the interspecific F_1 hybrid, use of bridge species, and, most importantly, embryo rescue. Various measures to crossability barriers were examined by several workers and are summarized in Table 8.4.

8.7.1 Embryo Rescue

With the improvement of embryo rescue and ovule culture, crossability success has been greatly enhanced in *Vigna* species. This is particularly important in those situations where the reason of incompatibility lies post-fertilization such as endosperm abortion. Barone et al. (1992) observed that the embryos and endosperm in the cross between *V. vexillata* and *V. unguiculata* degenerated within 5–8 days after pollination. Using embryo rescue, successful crossing could be accomplished in *V. mungo* × *V. umbellata* (Biswas and Dana 1975; Chen et al. 1983). Singh et al. (2003) also produced successful hybrids between *V. radiata* and *V. umbellata*, and the hybrids possessed intermediate morphology with MYMV resistance. Recently, Chaisan et al. (2013) successfully obtained interspecific hybrids between *V. radiata* (cv. Kamphaeng Saen 2) and *V. umbellata* (cv. Miyazaki) by rescuing the 12-day-old embryos on MS medium supplemented with 1 mg/L IAA, 0.2 mg/L kinetin, and 500 mg/L casein hydrolysate. Nevertheless, for successful recovery of hybrids through embryo rescue, selection of embryo at proper stage is the key as the older embryos tend to be more responsive to tissue culture procedures.

Table 8.4 Methods to overcome crossability barriers in food legumes

Method	Cross-combination	Reference
Reciprocal crosses	*V. radiata* × *V. mungo*	Verma and Singh (1986); Ravi et al. (1987)
Growth regulators	*V. radiata* × *V. umbellata*	Gupta et al. (2002)
Embryo rescue	*V. radiata* × *V. unguiculata*	Tyagi and Chawla (1999)
	V. radiata × *V. umbellata*	Chaisan et al. (2013)
	V. mungo × *V. radiata*	Gosal and Bajaj (1983a, b)
	V. radiata × *V. trilobata*	Sharma and Satija (1996)
	V. radiata × *V. radiata* var. *sublobata*	Sharma and Satija (1996)
	V. marina × *V. luteola*	Palmer et al. (2002)
	V. glabrescens × *V. radiata*	Chen et al. (1990)
	V. vexillata × *V. unguiculata*	Gomathinayagam et al. (1998)
	V. unguiculata × *V. mungo*	Shrivastava and Chawala (1993)
Explant culture of hybrids (cotyledonary node)	*V. radiata* × *V. mungo*	Avenido et al. 1991)
Chromosome doubling using colchicine	*V. radiata* × *V. mungo*	Pande et al. (1990)
	V. radiata × *V. trilobata*	Dana (1966)
	V. radiata × *V. umbellata*	Chaisan et al. (2013)
Use of bridge species	(*V. mungo* × *V. radiata*) × *V. angularis*	Gupta et al. (2002)

Modified from Kumar et al. (2011)

8.7.2 Irradiation Treatment and Reciprocal Crossing

Irradiation techniques have been successful in recovering fertile plants in F_1s and subsequent generations in interspecific crosses in many crop species. Pandiyan et al. (2008) recorded increased pod set in interspecific crosses between *V. radiata* and *V. umbellata* developed from gamma ray-irradiated parental lines. Similarly, reciprocal differences have also been found to be very common and can be due to chromosomal imbalance in the endosperm, the role of sperm nucleus in differential endosperm development, or the alteration of endosperm development by pollen by affecting antipodal cells which presumably supply nutrients during early endosperm development (Beaudry 1951). If disharmony between genome of one species and cytoplasm of the other is a cause of fertilization barrier, reciprocal crosses can be successful to recover hybrids. For example, while *V. mungo* × *V. radiata* cross was unsuccessful, its reciprocal cross, *V. radiata* × *V. mungo*, produced successful hybrids (Verma and Singh 1986; Ravi et al. 1987). Interspecific hybridization between *V. nakashimae* and *V. angularis* was successful in both directions and viable seeds were produced, while *V. riukinensis* produced successful hybrids when used as male parent only with *V. umbellata* (Siriwardhane et al. 1991). In general, a cross using a species with higher chromosome number as a female parent is more successful than its reciprocal.

8.7.3 Use of Bridge Species

Transfer of useful genes from secondary and tertiary gene pools cannot be usually accomplished through direct hybridization between cultivated and wild species. In such asses, sometimes the involvement of a third species as a bridge species can be successful for introgression of alien genes. For example, triticale (× Triticosecale) has been very commonly used to transfer alien genes from rye (*Secale cereale*) into wheat (*Triticum aestivum*). In cultivated *Vigna* species, no reliable sources of resistance have been reported till date for transfer of a notorious storage pest, bruchid. Transferring resistance to bruchid from wild *Vigna s*pecies is difficult due to cross-incompatibility. However, by using the bridge species, *V. nakashimae*, bruchid resistance has been successfully transferred from *V. umbellata* to azuki bean (Tomooka et al. 2000, 2003). Similarly, for transferring resistance genes from *V. vexillata* to cowpea, *V. davyi* was used as a bridge species, though the hybrid resulting from the cross between *V. vexillata* and *V. davyi* were partially fertile (Fatokun 1991).

8.7.4 Use of Growth Hormones

In distant crosses, post-pollination application of growth regulators such as gibberellic acid (GA3), naphthalene acetic acid (NAA), kinetin, or 2,4-D (2,4-dichlorophenoxyacetic acid), singly or as mixture, has been found to be helpful to the developing seeds by facilitating division of hybrid zygote and endosperm. In vivo hormonal treatments have also greatly helped in recovery of interspecific hybrids in *Vigna*. A true breeding *V. mungo* × *V. radiata* derivative was reciprocally crossed with *V. angularis*, and the pollinated pistils were treated with GA3 after 24 and 78 h of pollination and resulted in higher seed set.

8.7.5 Polyploidization

Increasing chromosome number of one or both the species, particularly in case where the two species involved in crossing have different ploidy level, can also enhance crossability between the two species. Ploidy level induction of plant cells by colchicine treatment is a useful technique in plant breeding aiming at enhancing biomass yield and resistance to stresses (Glowacka et al. 2010; Wu et al. 2012), as well as helping in resolving interspecific hybrid sterility problems (Miyashita et al. 2009). Using this technique, successful crosses have been attempted between *V. radiata* × *V. mungo* (Pande et al. 1990) and *V. radiata* × *V. trilobata* (Dana 1966). In the study by Chaisan et al. (2013), the hybrid sterility problem between the interspecific hybrids obtained from the cross *V. radiata* (cv. "Kamphaeng Saen 2) × *V. umbellata* (cv. Miyazaki) was resolved by colchicine treatment applied at 2 g/L. Three out of twenty hybrid seedlings were successfully induced from diploid to tetraploid which were subsequently able to produce flowers and set pods normally.

8.8 Alien Gene Transfer in *Vigna* Through Distant Hybridization

Mung bean × urd bean crosses have been very routinely attempted by various researchers since the derivatives from mung bean × urd bean crosses exhibit many desirable features such as lodging resistance, synchrony in podding, and non-shattering pods (Reddy and Singh 1990). Derivatives from mung bean × urd bean crosses have also been reported to exhibit higher level of yellow mosaic disease resistance caused by mung bean yellow mosaic virus (MYMV) (Gill et al. 1983). Besides this, traits such as long pods, number of seeds per pod, and erect plant type may be transferred from mung bean, while sympodial bearing and multiple clusters per peduncle may be transferred from urd bean into mung bean (Fig. 8.2). Singh and Dikshit (2002) successfully introgressed yield genes in mung bean from urd bean with 15–60 % yield advantage. Similarly, progenies from mung bean × rice bean and mung bean × *V. radiata* var. *sublobata* crosses were also recovered which exhibited high degree of resistance to MYMV (Verma and Brar 1996). Successful interspecific crosses between *V. unguiculata* and *V. vexillata* have also been reported. However, it was not confirmed through backcross breeding whether F_1 developed were true F_1 hybrids or not (Gomathinayagam et al. 1998). Tyagi and Chawla (1999) also reported successful crosses between *V. radiata* and *V. unguiculata* using in vitro culture techniques. Gibberellic acid treatment sustained the pods for 9–10 days, which were then used for embryo culture. About 10 % of total embryos resulted in plantlet formation. In this case also, the authors did not report further growth and culture of these plantlets, and therefore, it is not certain whether the crosses were true hybrids. A large number of promising material with novel traits in both mung bean and urd bean have been developed. The variability generated through these crosses for different agronomic traits is unique because such extreme types are not available in the collection of either mung bean or urd bean germplasm (Singh and Singh 1998; Singh and Dikshit 2002, Aditya Pratap unpublished data). The derivatives from mung bean × urd bean crosses exhibit many desirable features such as lodging resistance, synchrony in podding, and non-shattering habit (Reddy and Singh 1990). Singh and Dikshit (2002) were successful in introgressing yield genes in mung bean from urd bean with 15–60 % yield advantage. Similarly, progenies from mung bean × rice bean and mung bean × *V. radiata* var. *sublobata* hybrids having high degree of resistance to MYMV were also recovered (Verma and Brar 1996). The major post-harvest constraint of food legumes is susceptibility to bruchids (*Callosobruchus chinensis* L.) that eat seeds in storage. One accession of wild mung bean (*Vigna radiata var. sublobata*) exhibited complete resistance to azuki bean weevils and cowpea weevils (Fujii et al. 1989) which has been successfully used in breeding program (Tomooka et al. 1992). *Vigna mungo* var. *silvestris* is also reported to be immune to bruchids (Fujii et al. 1989; Dongre et al. 1996).

Although successful transfer of many desirable traits has been successfully accomplished in *Vigna* species from wild genetic resources, the actual release of new cultivars from distant crosses is scanty. Low fertility level in early generations allows only a limited recombination and usually leaves a small population for

Fig. 8.2 Some promising recombinants obtained from crosses between *V. radiata* × *V. mungo*—(**a**) high temperature tolerant; (**b**) more number of pods/plant; (**c**) long pod length; (**d**) more number of pods per cluster; (**e**) pod bearing from base of the plant—and *V. mungo* × *V. silvestris*, (**f**) Increased number of pod-bearing branches; (**g**) dwarf and erect plant type

selection. In India, three mung bean cultivars, viz., HUM 1, Pant Moong 4, and IPM99-125, and one urd bean cultivar, Mash 1008, have been developed from mung bean × urd bean crosses. These cultivars have improved plant types in addition to high resistance to yellow mosaic disease and synchronous maturity. While HUM 1 developed by Banaras Hindu University, Varanasi, has been released for cultivation in the Central and South Zones of India, Pant Mung 4 has been developed by G.B.

Fig. 8.3 Some outstanding derivatives obtained from *V. radiata* × *V. mungo* crosses—(**a**) IPM 99–125, a popular mung bean variety released for cultivation in the North East Plain Zone of India; (**b**, **c**) extra early maturing mung bean lines IPM 205–7 and IPM 409–4 developed from *V. radiata* × *V. mungo* cross-derived parental genotypes

Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India, and this variety has been released for the North East Plain Zone of the country. IPM 99–125 has been developed and released in 2004 for the North East Plain Zone of India by the Indian Institute of Pulses Research, Kanpur, and currently is one of the most popular varieties of mung bean in India (Fig. 8.3). In urd bean, Mash 1008 was developed by Punjab Agricultural University, Ludhiana, India, and was released for the Northwest Plain Zone of India in 2008. Using the above varieties developed from distant crosses, some more promising material has also been generated.

For example, using IPM 99–125 as one of the parents, genotypes IPM 02–1 and IPM 03–1 were developed in mung bean at IIPR, Kanpur, which were further used in the development of two extra early mung bean genotypes, IPM 205–7 and IPM 409–4, which mature in 46–48 days (Pratap et al. 2013a, Fig. 8.3). Both these genotypes are the earliest maturing materials in mung bean reported till date and will help in horizontal expansion of mung bean cultivation in India, especially during the spring/summer season in the northern India as well as in rice fallows in peninsular India. The major constraint for expansion of area in green gram as a summer crop in rice-wheat system is a short-season window with very hot weather (maximum temperatures touching 44 °C) conditions. Similarly during rainy season, the crop invariably witnesses rains at the time of pod maturity, leading to deterioration of seed quality and pre-harvest sprouting (Singh et al. 2011). Short-duration mung bean crop can avoid the adverse effects of terminal heat stress during summer season and adverse effect of untimely rains at harvest time during rainy season. These lines can also be useful as donors for development of super early green gram genotypes as well as in related *Vigna* species. Recognizing their potential as a cultivar and also a donor, IPM 205–7 has been registered as INGR 11043 and IPM 409–4 as INGR 11044 by the National Bureau of Plant Genetic Resources (ICAR), New Delhi, India, both for extra early maturity (Pratap et al. 2013b).

8.9 Genetic Transformation

Like other large-seeded legumes, alien gene transfer through genetic transformation in *Vigna* species has also been difficult, mainly because of their recalcitrant nature to in vitro procedures. Nevertheless, in the last three decades, significant progress has been made towards development of reproducible protocols for generation of transgenic vignas that permit the expression of alien genes in cultivated background. Direct multiple shoot organogenesis from cotyledonary node and shoot tip of the embryonic axis or seedling have been more useful for regeneration in *V. radiata* (Sonia Saini et al. 2007), *V. mungo* (Saini and Jaiwal 2005), and *V. unguiculata* (Chaudhary et al. 2007). In *V. radiata*, Pal et al. (1991) were the first to recover primary transformants on kanamycin selection from cotyledons of *V. radiata* inoculated with *Agrobacterium tumefaciens*. Later, Mahalakshmi et al. (2006) were able to develop transgenic mung bean plants from primary leaf explant on hygromycin selection medium with a frequency of 2 %. Sonia Saini et al. (2007) generated transgenic plants carrying *Phaseolus vulgaris* α-amylase inhibitor gene on PPT selection with 105 % frequency.

In urd bean, initially, transformation frequencies as high as 23 % with LBA4404 and 10 % with EHA105 strains of *A. tumefaciens* have been reported from leaf discs (Karthikeyan et al. 1996). However, the transformed calli could not be regenerated to develop a shoot. Saini et al. (2003) were able to develop fertile transgenic plants from cotyledonary node explants inoculated with *A. tumefaciens*. Saini and Jaiwal (2007) further optimized the conditions for enhanced transformation of

cotyledonary node and generated stable transgenic plants with a frequency of 4.3 %. Similarly, Muruganantham et al. (2007) developed herbicide (Basta®)-tolerant urd bean plants using cotyledonary node and shoot tip explants and *A. tumefaciens* harboring a binary vector carrying *bar* and *uidA* genes. The plants expressed stable integration of the transgene which were inherited in Mendelian fashion. More recently, Bhomkar et al. (2008) introduced salt stress tolerance in *V. mungo* by transferring glyoxalase 1 (*gly1*) gene under a constitutive Cestrum Yellow Leaf Curling Virus (CmYLCV) promotor. The T1 plants showing *gly1* activity survived and set seeds under NaCl stress.

In *V. unguiculata*, Sahoo et al. (2000) reported the recovery of chimeric transgenic plants under kanamycin selection using shoot apices. However, Popelka et al. (2006) first demonstrated stable transformation and expression of two cointegrated genes, *bar* and *uidA* in the progeny of transgenic plants with very low and varying frequencies. Later, Chaudhary et al. (2007) using the direct shoot regeneration protocol from the cotyledonary node explants improved the efficiency of transformation from 0.1 % (reported by Popelka et al. 2006) to 1.9 %. Recently, Solleti et al. (2008) successfully introduced bean α-amylase inhibitor-1 (*α-AI-1*) gene into cowpea to transfer bruchid (*Callosobruchus* spp.) resistance. The transformation protocol developed in this study did not appear to be variety specific and presented a high frequency of transformants following Mendelian segregation. The system comprised of biolistic transformation of meristems using gus as a reporter and novel selection regime based on the use of the herbicide imazapyr.

In *V. angularis*, stable transformants were developed using *A. tumefaciens*-mediated gene transfer to epicotyl explants and subsequent selection on medium containing kanamycin (Yamada et al. 2001), or hygromycin (El-Shemy et al. 2002) or PPT (Khalafalla et al. 2005). Agroinfection of epicotyl explants was also used to transfer *α-AI-1* and D6 fatty acid desaturase genes to impart resistance to bruchids (Yamada et al. 2005) and to produce polyunsaturated fatty acids (Chen et al. 2005).

8.10 Conclusions and Future Prospects

In the past, remarkable progress has been made towards the development of high yielding, stress-resistant, and input-responsive varieties in different *Vigna* crops using the cultivated germplasm. The impact of these varieties has been well realized by an increase in their productivity. However, there are still a number of areas which need attention to bring these crops in the mainstay of agricultural production. A major thrust is required on the incorporation of resistance to major diseases (mosaics, leaf spots, mildew) and insect-pests (thrips, jassids, borers, aphids, bruchids), tolerance to pre-harvest sprouting, and improved grain and nutritional quality. For many of these stresses, resistance level is not high in cultivated germplasm, and therefore, identification of diverse germplasm sources from wild species is required for transfer of important agronomic traits. Alien genes for several other traits such as photo- and thermo-insensitivity erect plant types for mechanical

harvesting, and resistance to pod shattering should also be looked for in the wild germplasm. Incorporation of earliness and development of short-duration varieties having distinct vegetative and reproductive phase are desirable for fitting them in cereal-based cropping systems as well as in those areas where these can be effectively taken as a catch crop, which will further increase their area and productivity (Pratap et al. 2013a). Many of the useful alien genes in wild species are expected to be different from those of the cultivated species and are thus useful in broadening the base of cultivated species. Recently, QTLs have also been identified for yield traits in wild species which may enhance agronomic and market values of cultivated varieties. Identification of high crossability genes in *Vigna* can bring non-crossable species within the ambit of alien gene transfer technology. Continuing advances in wide crossing techniques such as embryo culture and development of novel crossing strategies will further make wild gene pools of many crops even more accessible.

At the same time, much more efforts are needed towards establishment of universal genetic transformation protocols and in vitro regeneration techniques. To counter the possibility of insect resistance development to Bt toxin, gene pyramiding strategy needs to be adopted through sexual hybridization as well as genetic transformation. The continuing advances in structural genomics and genetic engineering will result in new strategies for alien gene introgression. The modern tools of molecular biology such as monoclonal antibodies, RNA-mediated gene silencing, and in situ hybridization using various DNA probes may further make it possible to study the mechanism of switching on/off of various genes in fertilized ovules as well as track the levels and movements of growth substances in developing seeds. Integrated breeding using conventional and genomic tools and alien gene detection through molecular and cytogenetic approaches will definitely help in successfully employing the alien gene transfer technologies for the genetic amelioration of various *Vigna* species.

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Chapter 9
Lentil

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Abstract Lentil (*Lens culinaris* Medik subsp. *Culinaris*) is an important cool season food legume grown over a large area in the Indian subcontinent, west Asia, some parts of Africa and southern Europe. Keeping in view its great demand for nutritious seeds, efforts are underway in different research institutes for development of improved plant types with high yielding ability, disease and insect pest resistance, and seed weight. Improvement of grain quality and nutrition are also among the import breeding objectives. While the cultivated germplasm is being utilized for addressing these objectives, even greater genetic variability has been observed in exotic germplasm and wild accessions. A better understanding of the pre- and postfertilization barriers and use of in vitro techniques and hormonal manipulations has improved the possibilities of obtaining viable and fertile interspecific hybrids using these wild genetic resources. Consequently, efforts have been made to transfer alien genes from wild species, viz., *L. culinaris* ssp. *orientalis* and *L. ervoides* to cultivated species. Despite these developments, strategies are still need to be developed to employ distant hybridization and alien gene introgression as a routine practice in genetic improvement of lentil. Further, a lot still requires to be done at molecular levels and gene transfer across genome boundaries through genetic transformation in lentil.

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9.1 Introduction

Lentil (*Lens culinaris* Medik subsp. *culinaris)* is an important cool season pulse crop grown worldwide including west Asia, the Indian subcontinent, Ethiopia, North Africa and to a lesser extent in southern Europe. It is rich source of protein (24–28 %) with an abundance of lysine, which makes it a good supplement with cereals for balancing the human diet. Its seed provides minerals and vitamins for human nutrition and straw for animal feed. Ability of lentil plant to fix atmospheric nitrogen and carbon sequestration improves soil health. Both small- and large-seeded lentils are grown in different parts of the world. Small-seeded lentils are produced especially in Turkey, Indian subcontinent, and Australia, while large-seeded green lentils are produced by Canada and USA, mainly for the purpose of export. Lentil is generally cultivated on poor soils under rainfed conditions by marginal farmers with minimum care and hence records poor yield. Further this crop is affected by various fungal, bacterial, and viral diseases and parasitic weed menace at various growth stages (Dita et al. 2006) leading to severe yield losses. Being rich in protein, several insect pests also cause yield losses to food legumes, both under field conditions and in storage (Clement et al. 1994, 1999). Among abiotic stresses, drought, temperature extremities, and edaphic problems (salinity and mineral toxicities) have great bearing on their harvestable yield (Stoddard et al. 2006).

It is well known that wild species are a rich reservoir of useful alien genes, which are no longer available within the cultivated gene pool (Hawkes 1977; Doyle 1988; Tanksley and McCouch 1997; Kumar et al. 2011). Therefore, continuous efforts have been underway to collect and conserve wild relatives of various food legume crops including lentil in the national and international gene banks (Plucknett et al. 1987; FAO 1996). Over the years, ICARDA has collected and conserved 587 accessions representing six wild *Lens* species from 26 countries. In the past, efforts have been made to search for genes imparting resistance to these stresses and other traits within the cultivated species, and their wild relatives. Success of introgression of alien genes from wild relatives has been achieved for few diseases and insect pests, which are controlled by major gene(s) (Knott and Dvorak 1976; Stalker 1980; Ladizinsky et al. 1988; Prescott-Allen and Prescott-Allen 1986, 1988; Hajjar and Hodgkin 2007). Further, the advances in tissue culture techniques have also been utilized. Now these techniques have opened new ways and helped in successful alien gene introgression in lentil. Extensive pre-breeding efforts have been made worldwide using those wild species, which carry useful alien genes for improving yield, quality, and stress resistance. This chapter summarizes the progress, achievements, and impacts of alien gene introgression in lentil.

9.2 Centers of Diversity

Distribution of wild species and an overlap of both wild and cultivated lentils in the Turkey-Cyprus region has supported that Southwest Asia or Near East or Mediterranean area is the primary center of diversity of the cultivated species, *Lens culinaris* (Cubero 1981). Earlier, the eastern border of Southwest Asia (i.e., region between Afghanistan, and Turkistan) was also considered as possible center of origin due to the presence of highest proportion of endemic varieties (Barulina 1930); later on, this region has been better explained as secondary center of diversity.

9.3 Crop Systematic and Species Relationships

Lentil belongs to subfamily Papilionoideae of legume. The species belonging to this subfamily are distributed into four clades: (1) the dalbergioid clade (it is more basal and mostly tropical), (2) the genistoid clade (Moretzsohn et al. 2007), (3) the haseoloids/millettioid clade, and (4) the galegoids/hologalegina clade. Lentil, which is a temperate or cool season legume, is the member of inverted repeat loss subclade (i.e., presence of a lost copy of the inverted repeat in the chloroplast genome of most angiosperms) of galegoids/hologalegina clade. Other members of this subclade are alfalfa (*M. truncatula*), chickpea (*Cicer arietinum*), faba bean (*Vicia faba*), and pea (*Pisum sativum*). A relationship based on molecular markers showed high-level synteny of lentil genome with the genome of *Medicago truncatula* and pea (Weeden et al. 1992; Phan et al. 2007).

Traditionally, the relationships among species have been established on basis of morphological characters and crossability among the species. This helped to establish their species or subspecies status. Free crossability of *L. odemensis* with *L. culinaris* subsp. *culinaris* and *L. culinaris* subsp. *orientalis* was identified as separate taxa from *L. nigricans* and *L. nigricans* with two subspecies, *nigricans* and *ervoides* (Ladizinsky et al. 1984). However, pollen and pistil morphology established close relationship between *L. odemensis* and *L. nigricans*, and these two species showed relationship with *L. culinaris* subsp. *orientalis. L. ervoides* was loosely related to all these taxa (Fratini et al. 2006). The specific status of *L. tomentosus* (Ladizinsky 1997) is still debatable because this species only separated from *L. culinaris* subsp. *orientalis* on the basis of tomentose as opposed to glabrous pods and by the presence of a small, asymmetrical, minutely satellited chromosome. *L. orientalis* is clearly the wild progenitor of cultivated species, and hence this was considered as subspecies of the cultivated species *L. culinaris* (Harlan and de Wet 1971). Based on morphological, biochemical, and molecular markers, *L. odemensis* and *L. tomentosus* have also been considered as the subspecies of *L culinaris* (Ferguson et al. 2000). Taxonomically, specific status of a species or subspecies of genus *Lens* is always confused. However, based on SDS-PAGE, ITS, and cytoplasmic DNA markers studies, the genus *Lens* consists of six main species (Cubero et al. 2009)

and two subspecies including the cultivated lentil, *L. culinaris* Medik., and it's subspecies *culinaris* and its wild progenitor *orientalis* (Boiss.) Ponert, *L. odemensis* Ladiz., *L. ervoides* (Brign.) Grande, and *L. nigricans* (M. Bieb.) Godr (Ladizinsky 1993) and two species recognized later, *L. tomentosus* Ladiz. and *L. lamottei* Czefr (Van Oss et al. 1997). Barulina (1930) described *microsperma* and *macrosperma* as two subspecies of cultivated lentil species on the basis of seed size. These two subspecies overlap to variable extent with the known wild lentils and are clearly intermixed. Easy cross-compatibility of *L. odemensis* with *L. culinaris* could have generated the genetic raw material for western lentils having bigger seeds, a high number of large leaflets and calyx teeth longer than corolla. The reports demonstrate that *L. orientalis* and *L. odemensis* forms are the most likely candidates as companion weeds of the cultigen, and disruption selection has only differentiated *microsperma* and *macrosperma* types during the course of evolution (Cubero et al. 2009).

9.4 Crossability Groups and Gene Pool

The information generated on the basis of crossability between the crop species and the subsequent cytogenetic, phylogenetic and molecular studies have been used to characterize the crop species into different gene pools (Harlan and de Wet 1971). The different species of lentil have been grouped into primary (*Lens culinaris* ssp. *culinaris*, *L. culinaris* ssp. *orientalis*, *L. odemensis*), secondary (*L. ervoides*, *L. nigricans*), and tertiary gene pools (*L. lamottei*, *L. tomentosus*) (Ladizinsky et al. 1984; Ladizinsky 1999; Muehlbauer and McPhee 2005). Hancock (2004) grouped lentil species in three groups only on the basis of crossing and placed inter-crossable species *L. nigricans*, *L. ervoides*, and *L. lamottei* in secondary pool or Group II. Crossing of Group I species with *L. nigricans* produces nonviable seed in hybrids due to irregular meiosis (Ladizinsky et al. 1984, 1985). However, the use of embryo rescue can produce viable seed in hybrids derived from crossing between *L. culinaris* and *L. ervoides* (Ladizinsky et al. 1985). They also put *L. tomentosus* in Group III or tertiary gene pool as a single species group, and this species does not produce viable seed in hybrids derived by crossing with other group of species.

For making genetic improvement by generating new variability, useful genes identified in the primary gene pool have readily been used for crop improvement. However, frequency of occurrence of these useful genes have been observed much more in the species belonging to secondary and tertiary gene pools (Collard et al. 2001; Tullu et al. 2006). Therefore, more efforts are required on deployment of novel techniques for using these gene pools for the improvement of lentils. Fratini and Ruiz (2006) suggested that hybrids between *L. culinaris* ssp. *culinaris* and *L. nigricans*, *L. ervoides*, and *L. odemensis*, developed through embryo rescue, can be viable with a rate of 3–9 %. Based on these observations, *L. odemensis* has been considered to be a member of secondary gene pool, and *L. nigricans* and *L. ervoides* were classified in the tertiary gene pool (Ladizinsky 1993). Further, Cubero et al. (2009) also suggested to place *L. odemensis* in secondary gene pool,

while *L. nigricans* and *L. ervoides* can also be part of secondary gene pool as hybrids could be obtained by means of embryo rescue. They also suggested that there is a need to study hybridization in order to establish place of *L. tomentosus* and *L. lamottei* in secondary or tertiary gene pool.

9.5 Traits of Economic Importance in Wild Species

Wild relatives and exotic landraces have a repository of useful genes controlling the important traits. The wild species show large genetic diversity for morphological and seed traits (Fig. 9.1a, b). Lens gene pool consists of many wild relatives offering resistance to biotic (Ahmad et al. 1997a, b) and abiotic stresses (Hamdi et al. 1996). ICARDA maintains more than 500 landraces and wild species of Mediterranean region. Accessions of different wild species have been screened for agro-morphological traits besides key biotic and abiotic stresses at ICARDA, Aleppo, Syria; IIPR, Kanpur; and other advanced research institutions (Table 9.1). Accessions belonging to *L. odemensis* and *L. ervoides* showed drought tolerance (Hamdi and Erskine 1996; Gupta and Sharma 2006), while cold tolerance and earliness have been observed in *L. culinaris* ssp. *orientalis* (Hamdi et al. 1996). Combined resistance to ascochyta blight and fusarium wilt (ILWL 138) or anthracnose diseases (IG 72653, IG 72646, IG 72651) have also been identified (Bayaa et al. 1995; Tullu et al. 2006). Gupta and Sharma (2006) reported genetic variability for yield attributes and biotic and abiotic stresses among 70 accessions of four wild species/subspecies (*L. culinaris* ssp. *orientalis*, *L. odemensis*, *L. ervoides*, and *L. nigricans*). As the result, donors for resistance to powdery mildew [*L. culinaris* ssp. *orientalis* (ILWL 200, *L. nigricans* (ILWL 37)], rust, and wilt resistance have been found in all the above four species and also for drought tolerance in *L. nigricans* and seeds per plant in *L. culinaris* ssp. *orientalis* (ILWL 90). Multiple resistances have also been reported in some accessions of *L. nigricans* (ILWL 37) and *L. culinaris* ssp. *orientalis* (ILWL 77), and hence it can be a useful source of alien resistance genes. Studies have also showed resistance to sitona weevil more frequently found in *L. odemensis*, followed by *L. ervoides*, *L. culinaris* ssp. *orientalis*, and *L. nigricans* (El-Bouhssini et al. 2008). Indian Institute of Pulses Research, Kanpur, India, has maintained 117 landraces of Mediterranean region and 298 accessions of wild species (Fig. 9.1). Among these accessions, 91 landraces and 88 accessions of four wild species have been evaluated for morphophysiological traits under the field conditions as well as at hot spots for rust. Accessions belonging to *L. culinaris* ssp. *orientalis* (ILWL 147, ILWL 150, ILWL 189, ILWL 192, ILWL 230, ILWL 231, ILWL 248, ILWL 355, ILWL 365, ILWL 371, ILWL 425, IG135392, ILWL 118, ILWL 99), *L. odemensis* (ILWL 163), and *L. ervoides* (ILWL 30) and 14 accessions of landraces [IG 9, IG 13, IG 5069 (Jordan), IG 163, IG 195, IG 482, IG 485, IG 70208 (Turkey), IG 69546 (Ethiopia), IG 70238 (Egypt), and IG 71710, IG 73717, IG 73789, IG 73802] were observed to be highly resistant to rust disease because they were identified free from rust symptoms at Dhaula Kuan, India, which is a hot spot

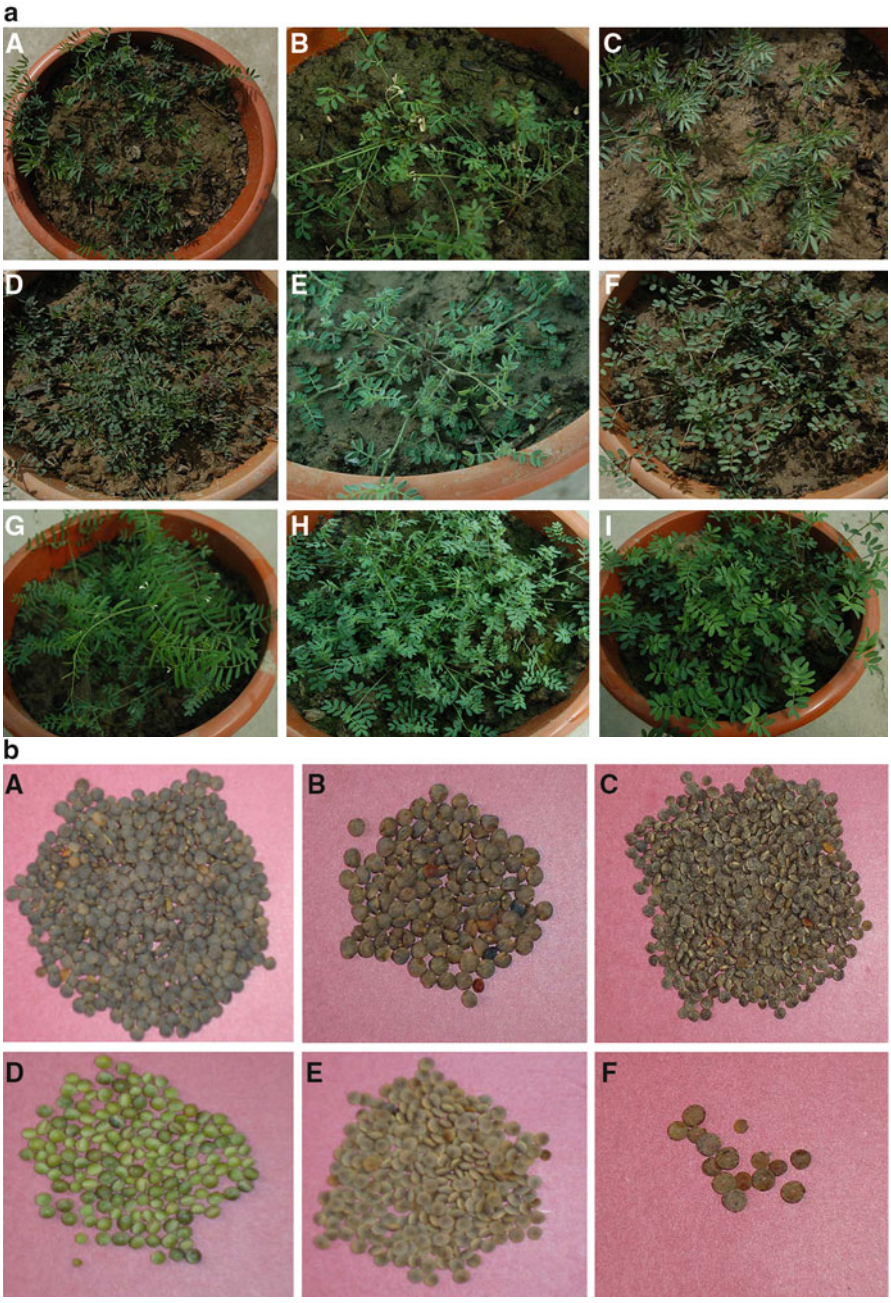

Fig. 9.1 (**a**) Morphophysiological variation in wild accessions of lentil. (*A*) *L. ervoides*, *(B) L. culinaris* ssp. *odemensis*, *(C) L. nigricans*, *(D) L. culinaris* ssp. *odemensis*, *(E) L. culinaris* ssp. *orientalis*, *(F) L. nigricans*, *(G) L. culinaris* ssp. *odemensis*, *(H) L. culinaris* ssp. *orientalis*, and *(I) L. culinaris* ssp. *orientalis.* (**b**) Variation is seed shape and color in wild accessions of lentil. (*A) L. nigricans*, *(B) L. Culinaris* ssp. *odemensis*, *(C) L. ervoides*, *(D) L. culinaris* ssp. *odemensis*, *(E) L. lamottei*, and *(F) L. nigricans*

Table 9.1 Traits of economic importance in wild germplasm for introgression of alien genes in lentil

Useful trait(s)	Wild species	Reference
Anthracnose resistance	*Lens ervoides*, *L. lamottei*, *L. nigricans*	Tullu et al. (2006)
Ascochyta blight resistance	*L. ervoides*, *L. culinaris* ssp. *orientalis*, *L. odemensis*, *L. nigricans*, *L. montbretti*	Bayaa et al. (1994); Tullu et al. (2010)
Fusarium wilt resistance	*L. culinaris* ssp. *orientalis*, *L. ervoides*	Bayaa et al. (1995); Gupta and Sharma (2006)
Powdery mildew resistance	*L. culinaris* ssp. *orientalis*, *L. nigricans*	Gupta and Sharma (2006)
Rust resistance	*L. culinaris* ssp. *orientalis*, *L. ervoides*, *L. nigricans*, *L. odemensis*	Gupta and Sharma (2006); Ashwani K. Basandrai and Jitendra Kumar (unpublished)
Drought tolerance	*L. odemensis*, *L. ervoides*, *L. nigricans*	Hamdi and Erskine (1996), Gupta and Sharma (2006)
Cold tolerance	*L. culinaris* ssp. *orientalis*	Hamdi et al. (1996)
Yield attributes	*L. culinaris* ssp. *orientalis*	Gupta and Sharma (2006)
Resistance to Orobanche	*Lens ervoides*, *L. odemensis*, *L. orientalis*	Ferna´Ndez-Aparicio et al. (2009)
Resistance to sitona weevils	*L. odemensis*, *L. ervoides*, *L. nigricans*, *L. culinaris* ssp. *orientalis*	El-Bouhssini et al. (2008)
Resistant to seed bruchids	*L. culinaris* Medikus subsp. *orientalis* (Boiss.) Ponert, *L. nigricans* (M. Bieb.) Godr., and *L. lamottei* Cezfr.	Laserna-Ruiz et al. 2012

for lentil rust (Ashwani K. Basandrai and Jitendra Kumar, unpublished). Similarly, a very wide range of genetic variability for ten morphophysiological traits including seed weight and number of seeds per pod has been observed in 88 wild accessions of lentil belonging to five wild species of lentil (Pratap et al. 2014).

9.6 Limitations in Use of Alien Gene Introgression

It is well recognized that gene transfer through wide crosses is a long and tedious process for the species having cross incompatibility with cultivated species due to the lack of homology between chromosomes of participating species in the cross. Pre- and post-zygotic crossability barriers occur between wild and cultivated species. Utilizing wild gene pool in breeding program may also be constrained by collection gaps in wild species with no information on genome relationship, poor/limited screening of wild species, linkage drag, and genetic complexity of the traits. Therefore, improvement through distant hybridization often takes longer time in order to recover genotypes associated with acceptable agronomic background and thus requires a long-term approach in place.

Reproductive isolation, embryo or endosperm abortion, hybrid sterility, and limited levels of genetic recombination are significant obstacles for introgression of alien genes from wild germplasm at a larger extent in lentil breeding programs. These barriers can occur before or after the fertilization of male and female gametes because the pollen tube does not reach ovary or the male gametes do not fuse with female gametes (Chowdhury and Chowdhury 1983). Thus, strong crossability barriers limit utilization of wild gene pool for lentil improvement. In lentil, abnormal chromosome pairing between the participating genomes has been observed in the crosses of *L. culinaris*×*L. tomentosus* (Ladizinsky 1979). In some *L. culinaris*× *L. culinaris* ssp. *orientalis* crosses, development of endosperm was observed to be normal, while the hybrid embryo ceased growing (Ladizinsky 1993). In contrast, Abbo and Ladizinsky (1991) observed that the endosperm was either abnormal or lacking in *L. culinaris*×*L. culinaris* ssp. *orientalis* crosses. Hybrids showed varying degrees of fertility usually due to chromosome translocations and subsequent problems with chromosome pairing at meiosis in *Lens culinaris*×*L. nigricans* (Goshen et al. 1982; Ladizinsky et al. 1984). Fertility is often very low with little viable pollen produced in anthers and varies depending on the accession in *L. culinaris*×*L. culinaris* ssp. *orientalis* crosses from 2 to 69 % (Ladizinsky et al. 1984). These problems can occur in the F_1 and also persist in later generations causing partial or complete sterility. Albino seedlings can also occur in the F_1 generation and thus prevent hybridization success (Ladizinsky and Abbo 1993). Another common problem is that hybrid embryos cease to grow about 7–14 days after pollination due to endosperm degeneration and thus need rescuing in order to obtain viable hybrids (Ladizinsky et al. 1985; Ahmad et al. 1995). Hence, *L. culinaris*×*L. ervoides* or *L. culinaris*×*L. nigricans* crosses need embryo rescue techniques in order to develop mature hybrid plants (Cohen et al. 1984; Abbo and Ladizinsky 1991).

9.7 Strategies Used for Alien Gene Introgression

The better understanding of the pre- and postfertilization barriers has improved the possibilities considerably to manipulate these processes towards the development of viable and fertile interspecific hybrids. During past several years, various measures have been used successfully to break the crossability barriers (Kumar et al. 2011), which have been discussed briefly below.

9.7.1 Use of Cross-Compatibility Between Wild and Cultivated Species

Release of new hidden variability present in the background of wild species depends upon the cross-compatibility of these species with the cultivated species. Therefore, during the past years, efforts have been made in lentil to study the

cross-compatibility of wild species with the cultivated species. It has been shown that *L. culinaris* ssp. *orientalis* and *L. odemensis* are crossable with cultivated lentil (Ladizinsky et al. 1984; Abbo and Ladizinsky 1991, 1994; Fratini et al. 2004; Fratini and Ruiz 2006; Muehlbauer et al. 2006), although the fertility of hybrids depends on the chromosome arrangement of wild parent (Ladizinsky 1979; Ladizinsky et al. 1984). Most accessions of *L. culinaris* ssp. *orientalis* cross readily with *L. culinaris*, and both are genetically isolated from the other species. *Lens nigricans* and *L. ervoides* are not readily crossable with the cultivated lentil using conventional crossing methods due to hybrid embryo breakdown (Abbo and Ladizinsky 1991, 1994; Gupta and Sharma 2005). Crosses are possible between *L. culinaris* and the remaining species, but they are characterized by a high frequency of hybrid embryo abortion, albino seedlings, and chromosomal rearrangements that result in hybrid sterility, if these seedlings reach maturity (Abbo and Ladizinsky 1991, 1994; Ladizinsky 1993; Gupta and Sharma 2005). Only four crosses have not resulted in formation of F_1 hybrids to date, viz., *L. culinaris* ssp. *orientalis* × *L. ervoides* and *L. culinaris* ssp. *orientalis* × *L. nigricans* (Ladizinsky et al. 1984), *L. culinaris* ssp. *tomentosus* × *L. lamottei* (van Oss et al. 1997), and *L. culinaris* ssp. *odemensis* × *L. ervoides* (Ladizinsky et al. 1984) though viable hybrids have been reported between cultivated species and *L. ervoides*, *L. odemensis*, and *L. nigricans* with the use of GA3 (Ahmad et al. 1995). Fratini et al. (2006) reported a high correlation between crossing success and phenotypic similarity based on pollen morphology and in vitro pollen length together with pistil and style length, indicating good predictor of hybridization success between different species.

9.7.2 Embryo Rescue

The advent of in vitro techniques such as embryo and ovule culture coupled with in vivo hormonal treatments has increased the scope of distant hybridization in lentil by overcoming the postfertilization barriers (zygotic abortion mechanisms). The possibility of increasing crossability also exists by predisposing crop embryos to alien endosperm and then using plants raised from those embryos to cross with the alien species. A two-step in vitro method of embryo-ovule rescue was developed to obtain successful distant hybrids by crossing the cultivated lentil with *L. ervoides* and *L. nigricans* (Cohen et al. 1984). However, the same technique used by other workers could not produce the hybrids (Ahmad et al. 1995; Gupta and Sharma 2005). Another study reported development of different protocol (Fratini and Ruiz 2006), which could be able to rescue the hybrid ovules 18 days after pollination. Fiala (2006) also obtained *L. culinaris* × *L. ervoides* hybrids using the Cohen et al. (1984) protocol, and one viable hybrid was also produced from a distant cross, *L. culinaris* ssp. *culinaris* × *L. lamottei*. Nevertheless, more efforts are required to develop the appropriate and efficient in vitro protocols that can be used routinely for rescuing immature hybrid embryos in order to make the alien gene introgression from wild species. The studies that uses embryo rescue to overcome crossability barriers in lentil have been listed in Table 9.2.

Table 9.2 Use of embryo rescue technique to overcome crossability barriers in lentil

Cross combination	Reference
L. culinaris × *L. orientalis*	Ladizinsky et al. (1985); Ahmad et al. (1995)
L. culinaris × *L. odemensis*	Goshen et al. (1982); Fratini and Ruiz (2006)
L. culinaris × *L. tomentosus*	Ladizinsky and Abbo (1993)
L. culinaris × *L. ervoides*	Cohen et al. (1984); Ahmad et al. (1995); Fiala (2006); Fratini and Ruiz (2006)
L. culinaris × *L. lamottei*	Fiala (2006)
L. culinaris × *L. nigricans*	Cohen et al. (1984); Fratini and Ruiz (2006)
L. orientalis × *L. odemensis*	Goshen et al. (1982); Ladizinsky et al. (1985)
L. orientalis × *L. tomentosus*	Ladizinsky and Abbo (1993); van Oss et al. (1997)

9.7.3 Use of Bridge Species

Direct hybridization between cultivated and wild species does not result in fertile hybrids, when useful genes are transferred from species belonging to secondary and tertiary gene pools. In this case, involvement of third species as bridge species has often been used for introgression of alien genes. In lentil, attempts have been made to transfer the alien genes from *L. lamottei* and *L. nigricans* to *Lens culinaris* using *L. ervoides* as a bridge species along with embryo rescue technique. This offers the possibility of transferring the genes for resistance to ascochyta blight and anthracnose to *L. culinaris* and broadening the resistance gene base in the cultivated species (Ye et al. 2002; Tullu et al. 2006).

9.7.4 Growth Hormones

It has been shown that post-pollination application of growth regulators such as gibberellic acid (GA3), naphthalene acetic acid (NAA), and kinetin or 2,4-dichlorophenoxy acetic acid (2,4-D) (dimethylamine) singly or as mixture may help to maintain the developing seeds from those interspecific hybrids, which may die due to small embryos. In lentil, use of GA3 has resulted in development of viable F_1s between cultivated species and *L. ervoides*, *L. odemensis*, and *L. nigricans* (Ahmad et al. 1995).

9.8 Pre-breeding and Alien Gene Introgression in Lentil

The wild relatives and exotic lines generate new variability through recombination breeding approach. The ultimate goal of wide hybridization is to transfer useful genes from alien species into cultivated species. It has been very successful in a few crops including lentil (Table 9.3). For example, resistance to anthracnose found in *Lens ervoides* germplasm is exploited in Canada by introgressing resistance genes into cultivated backgrounds (Fiala 2006; Tullu et al. 2006; Fiala et al. 2009). This successful

Table 9.3 Alien gene introgression for disease and agronomic traits in lentil

Wild species/exotic lines	Character/variety	References
Lens orientalis	Cold tolerance	Hamdi et al. (1996)
	Agronomic traits	Abbo et al. (1992), ICARDA (1995)
	Pods/plant, seed size	Jitendra Kumar (unpublished)
Lens ervoides	Anthracnose resistance, seed size	Fiala (2006); Tullu et al. (2006); Fiala et al. (2009); Tullu et al. (2011)
Precoz	High yields [Variety IPL 316: Sehore-74-3 × DPL58 (a derivative of Precoz)]	Jitendra Kumar (unpublished)

use of *Lens ervoides* holds promise as a source of genes for resistance to other diseases and possibly for plant habit, biomass production, and other important agronomic and marketing traits. Crossing of cv "Eston" (*L. culinaris*) with PI72815 and L01-827 (*L. ervoides*) was successful with the aid of embryo rescue. Production of hybrid seeds followed by F_2–F_7 seeds led to the development of two recombinant inbred populations (RILs) with varying degrees of sterility. However, among these RILs, several lines were observed as transgressive segregants for various agronomic traits including an 8 % increase in seed size. These lines could be useful as pre-breeding material for utilizing in lentil breeding program (Tullu et al. 2011). In India, a network work project in collaboration between the Indian Council of Agricultural Research (ICAR) and ICARDA funded by Department of Agriculture and Cooperation (DAC), Government of India, has been launched in 2010 for introgression of alien genes from unadapted germplasm and wild species in order to break the yield barriers in lentil. This project led to introgression the alien genes from wild species into the cultivated species using knowledge of cross-compatibility between wild and cultivated species. A segregating F_2 population comprising of 250 individuals was generated from crosses between the cultivated species and *L. orientalis*. This F_2 population showed a wide range of variability for agronomically important traits including days to 50 % flowering (61–98 days), secondary branches 2-9 pods/plant (2–432), biological yield/plant (1.08–50.83 g), grain yield/plant (0.10–20.74 g), and 100 seed weight (1.28–3.85 g). Transgressive segregants for the above traits were also identified from this population. Recently, Singh et al. (2013) made successful crosses between cultivated (*Lens culinaris* ssp. *orientalis*) and wild lentils (*L. culinaris* ssp. *orientalis, odemensis, lamottei* and *ervoides*) while effects of species groups, day length and temperature on crossability were clearly visible and a high level of heterosis was observed in F1 crosses for the important traits studied.

In the past, exotic line Precoz has been involved in lentil breeding program at Indian Institute of Pulses Research, Kanpur, India. As a result, a large number of breeding lines have been developed for seed size and earliness. These lines have been used further as pre-breeding material and crossed with locally adapted genotypes which have resulted in the development of a high yielding and large-seeded variety IPL 316. This variety has been released for cultivation in the central part of India including Madhya Pradesh, Chhattisgarh, and parts of Rajasthan and Bundelkhand region of Uttar Pradesh.

9.9 Conclusions and Future Prospects

The wild relatives and exotic materials have been important sources of alien genes controlling desirable traits for making genetic improvement. These genes can be helpful to develop improved cultivars with high productivity due to tolerance to various biotic and abiotic stresses. Many of the useful alien genes are expected to be different from those of the cultivated species and are thus useful in broadening the base of resistance to various stresses. During the past years, efforts have been made to transfer the alien genes from wild to cultivated species in lentil using conventional cross-compatibility approach as well as advanced tissue culture approach. These approaches could be able to transfer genes from species belonging to either primary gene pool or secondary gene pool with the aid of embryo rescue technique. However, still several limitations have restricted the mobilization of numerous desirable genes for abiotic and biotic stress from secondary and tertiary gene pools. Therefore, there is a need to make embryo rescue technique much more routine and also to study the wild species extensively to search for high-crossability genes as has been done in the case of Chinese Spring in wheat. Identification of such genes in lentil can bring uncrossable species within the ambit of alien gene transfer technology. The success rate of gene transfer in such wide crosses can be increased by the knowledge of the chromosome pairing mechanisms and their genetic control, by applying new strategies, systematic utilization of wild species in the breeding program, and proper utilization of pre-breeding materials generated from wide crosses. Use of advanced backcross QTL analysis will help to identify the favorable QTL in the background of wild species and their utilization in the breeding program. It will also help to minimize the transfer of undesirable linkage drag from wild species to cultivated species. Further, modern tools of molecular biology resulted through advances in structural genomics and genetic engineering will result in new strategies and increased success for alien gene introgression.

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Chapter 10
Brassica

Mayank Gautam, Xian-hong Ge, and Zai-yun Li

Abstract Wild crop relatives have been playing an important role in deciphering the plant genome and genetic improvement of the crop plants both qualitatively and quantitatively. They have been used in understanding the fundamental questions related to origin, evolution, phylogenetic relationships, cytological status, and introgression of nuclear and cytoplasmic genes for the genetic improvements of their domesticated counterparts and facilitating the innovation of many novel concepts while working on them directly or by using them. Owing to their high economic importance species of *Brassica* (monogenomic diploids, *B. nigra* (B genome, $n=8$), *B. oleracea* (C genome, $n=9$), and *B. rapa* (A genome, $n=10$) and digenomics, *B. carinata* (BC, $n=17$), *B. juncea* (AB, $n=18$), and *B. napus* (AC, $n=19$)) manifest many morphological variations and research applications that have been favorite of plant breeders. Oilseed brassicas are interesting breeding material since they have a complete range of breeding systems varying from complete cross-pollination to self-pollination. Both interspecific and intergeneric hybridizations have a great potential for creating new variability. Some of them are contributing as model plants for comparative crop genetics (*Arabidopsis thaliana*, *B. rapa*, etc.). Wild allies of *Brassica* have attracted breeders due to their enormous genetic, genomic, and breeding potential which can be harnessed for crop improvement, obtaining phytomedicines and nutraceuticals, bioenergy production, soil reclamation, and the phytoremediation of ecology or environment. The sexual and somatic wide hybrids, cytoplasmic sterile line, and addition lines raised between *Brassica* crop species and interspecific/intergeneric, intersubtribal, and intertribal members have not only lead to the widening of crop gene pool but have also assisted in breeding at local, regional, and global level by introgressing desirable traits to overcome unprecedented

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environmental changes and diseases. Significant increases in seed yield have been achieved in oilseed brassicas through the development of hybrid cultivars. This chapter emphasizes upon the progenies of wide hybrids with potential agronomic traits for *Brassica* breeding as well as the achievements and impacts of alien gene transfer in *Brassica*, achieved mainly through distant hybridization and somatic hybridization.

Keywords Alien species • *Brassica* species • Cytogenetics • Wide hybridization • Somatic hybrids • Addition lines • Erucic acid

10.1 Introduction

Brassicaceae (Cruciferae) is the fifth largest monophyletic family having approximately 3,700 species in 338 genera (Gómez-Campo and Prakash 1999; Prakash 2010). Economically, it contributes to 10 % of the world's vegetable crop production and approximately 12 % of the worldwide edible oil source besides being a promising potential source of biofuel. Canola is now the world's third largest source of vegetable oil (13 %), after soybean (32 %) and palm oil (28 %). The rapeseed production has witnessed a steady upward movement during the past 25 years, and presently, it contributes about 14 % of the global vegetable oils (Gupta and Pratap 2007). One of the spectacular achievements in *Brassica* research concerns the improvement in nutritional quality of oil and meal, primarily in *B. napus* and subsequently in other species and represents a classical example of plant breeding. The *Brassica* crops are unique because every plant part has been selected and manipulated to yield different products. They provide edible oils, condiments (seeds), and vegetables (roots, leaves, stem, and inflorescence). Crop brassicas lack many desirable traits. Enriching conventional germplasm with genes from the related germplasm and widening genetic base is a highly desirable approach. Majority of the species in this germpool are wild and weedy and distributed mostly in the Mediterranean phytochorea. This germplasm referred to as *Brassica* Coenospecies (Gómez-Campo 1999a, b) has the potential to exchange genetic material with crop brassicas to confer agronomic advantages. It comprises of 14 genera from three subtribes, viz., *Brassicinae*, *Raphaninae,* and *Moricandiinae* in the tribe *Brassiceae*. Major investigations on this germplasm were initiated by Manton (1932) who determined the chromosome numbers; Mizushima (1950a, b, 1968, 1980) hybridized wild and crop species to investigate intergenomic homoeology; and Harberd (1972) classified the germplasm into cytodemes. Many investigations dealing with morphology, molecular marker-based taxonomy, and intensive hybridizations have generated a worth of vast informations since the 1950s.

Toshitaro Morinaga carried out a comprehensive genome analysis of crop species (1928–1934) and proposed the diploid nature of *B. nigra*, *B. oleracea*, and *B. rapa* and allopolyploid evolution for *B. carinata*, *B. juncea*, and *B. napus.* Korean botanist Woo Jang-choon or Nagaharu (1935) working in Japan presented the

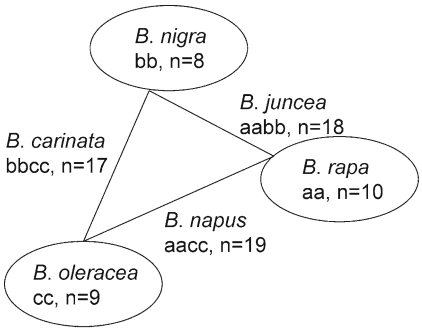

Fig. 10.1 Genomic relationship among the six cultivated *Brassica* species (1935) and showing the nucleolar dominance hierarchy in *Brassica* species bb>aa>cc

cytogenetic relationships among crop species in his famous U triangle (Fig. 10.1) consisting of three low chromosome monogenomic diploids—*B. nigra* (B genome, $n=8$), *B. oleraceae* (C genome, $n=9$), and *B. rapa* (A genome, $n=10$) and three high chromosome digenomics, i.e., *B. carinata* (BC, $n=17$), *B. juncea* (AB, $n=18$), and *B. napus* (AC, $n=19$) which evolved in nature through convergent alloploid evolution between any two diploid species and also experimentally demonstrated the allopolyploid evolution of *Brassica napus* synthesis. These relationships are further substantiated by cytogenetics, molecular analysis of nuclear and chloroplast DNA, and genomic and fluorescence in situ hybridization (Snowdon et al. 2003; Snowdon 2007), and the U triangle is now considered as a model system for investigating crop polyploidization (Lukens et al. 2006; Pires et al. 2006).

10.2 *Brassica* Crops Are Highly Polymorphic

Figure 10.2 showed the probable geographic centers of origin and domestication of different species of *Brassica* based on genetic diversity. *Brassica oleracea*, *B. rapa*, and *B. juncea* are highly polymorphic displaying a wide range of morphotypes; *B. nigra* is exclusively cultivated for the condiment mustard. The cultivated *B. oleracea* forms exhibit enormous morphological variability in leaf, stem, and inflorescence and are collectively referred to as cole crops—a term given by Bailey (1922), the American botanist and horticulturist in 1901. All these forms are sources of popular vegetables worldwide. Forms of *B. rapa* are variously termed as turnip rape (oilseed forms of Europe and Canada), *sarson* (oil seed forms of Indian subcontinent), and leafy vegetables (China and other Southeast Asian countries). *B. carinata*, the Ethiopian mustard, has a range of uses, e.g., edible oil, spices, medicinals, and vegetables. Its cultivation is restricted primarily to Ethiopia but also extends to Kenya. *B. juncea* (Indian or brown mustard) is a major source of edible oil in Indian subcontinent and Eastern European countries, of vegetables (leaf mustard) in China, and hot mustard condiment used in mayonnaise, salad dressing, and sauces in Europe, Canada, and America. *B. napus* is a major edible oilseed crop widely grown in Europe, Canada, China, and Australia. The development of canola as a crop can

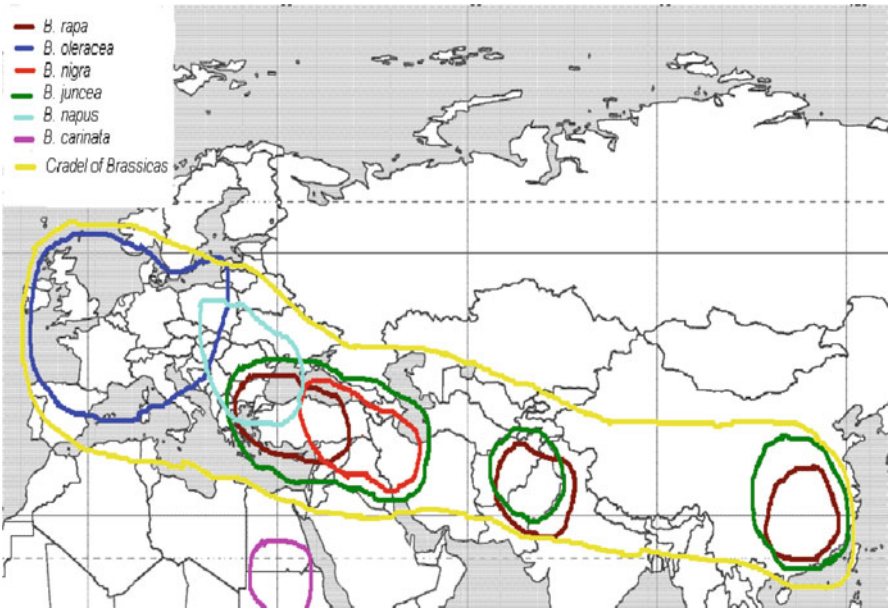

Fig. 10.2 Probable geographic centers of origin and domestication

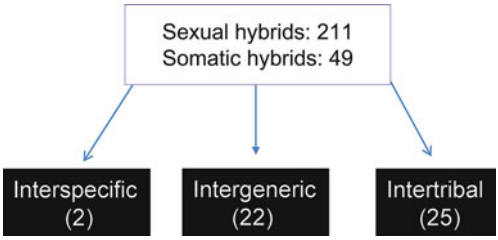

Fig. 10.3 Hybrids obtained between wild and *Brassica* crop series

be credited to the pioneering activity of Canadian *Brassica* breeder, R.K. Downey (Rakow 2000). In the last 50 years, several new fodder and vegetable types of *B. napus* using leafy and root forming forms of *B. rapa,* viz., ssp. *chinensis*, *pekinensis*, *narinosa*, *nipposinica,* and *rapa,* have been synthesized. The brassicas are important components to the cuisine of many cultures. These represent a valuable source of vitamin C, dietary fiber, and anticancer compounds (Fahey et al. 1997).

This chapter focuses on the wide hybridization of *Brassica* spp., (interspecific/intergeneric, intertribal (Fig. 10.3), sexual and somatic hybrids), their general cytological examination, and their implications in breeding, epigenetic control and conservation of wild germplasm.

With ever-increasing world population, enhancement of food production is a major necessity. Plant breeding is the purposeful manipulation of plant species in order to create desired plant types that are better suited for cultivation, giving better

yield and are disease resistant. Conventional plant breeding has been practiced for thousands of years since the beginning of human civilization; recorded evidence of plant breeding dates back to 9,000–11,000 years ago. Classical plant breeding involves crossing or hybridization of pure line, followed by artificial selection to produce plants with desirable traits of higher yield, nutrition, and resistance to diseases. With the advancement in genetics, molecular biology, and tissue culture, plant breeding is now increasingly being practiced by using molecular techniques.

The world is entering a period of unprecedented change in climate, and plant breeders must ensure that their breeding programs contain sufficient genetic diversity to respond to potential changes in the environment. In the last 70 years, many wild species in *Brassicaceae* (Prakash et al. 2009) have been used for generating wide hybrids because of their enormous desirable agronomic traits which can be used for improvement of crop species by the breeders and plant biologists. For example, *Orychophragmus violaceus* (L.) O. E. Schulz [syn. *Moricandia sonchifolia* (Bunge) Hook Fil.] is a member of the Brassiceae tribe. This species is cultivated as an ornamental plant in China, and its wild forms occur both in China and Korea (Luo et al. 1994), having super oil quality including natural zero-erucic acid; oleic, 20.32 %; high linoleic, 53.17 %; palmic, 14.31 %; linolenic, 4.76 %; and erucic acid, 0.94 % (Li et al. 1995, 1996, 1998, 2003, 2005; Ma et al. 2006; Ma and Li 2007; Wu et al. 1997; Xu et al. 2007a, b; Zhao et al. 2007, 2008; Ge et al. 2009). *Capsella bursa-pastoris* ($2n=32$) is a traditional vegetable, medicinal plant, natural double-low (erucic acid, glucosinolates) germplasm, and highly resistant to *Alternaria*, *Sclerotinia*, and cold (Chen et al. 2007). *Capsella rubella* ($2n=16$) is being sequenced in Europe. Comparison of genomes of different crop plants with *Arabidopsis* as a model has become a routine event in plant breeding. The only donor conferring the low glucosinolate in all *B. napus* varieties was "Bronowski" from Poland and low erucic acid from "Liho" in Germany. *Isatis indigotica* ($2n=14$) is a medicinal plant. Its roots are used as raw materials for preparing medicine to cure virus—cold. Extracts of roots and leaves impart resistant to bacteria and viruses (Du et al. 2009; Tu et al. 2008, 2009, 2010). *Lesquerella fendleri* is a valuable genetic resource for the rapeseed breeding for industrial purpose as it possesses high amounts of hydroxy fatty acids. In addition, the high tolerance to drought of *L. fendleri* is also useful for the genetic improvement of rapeseed (Du et al. 2008), especially due to thick glutinous polysaccharide layer on its seed coats. With the advancement of hybridization techniques, hormonal manipulations, genetic transformation, and embryo rescue, alien gene transfer has now become a more common practice involving larger numbers of crop species.

In the early nineteenth century, Sageret (1826) obtained intersubtribal hybrid (*Raphanus sativus* × *B. oleracea*) and Herbert (1847) raised interspecific hybrid (*B. napus* × *B. Rapa*) in *Brassica*. Initially, these hybridizations were used for cytological studies for understanding genomic homoeology. Afterwards, wide hybrids were routinely obtained for widening genetic base, introgressing nuclear genes that are valuable for providing tolerance to biotic and abiotic stresses in selected combinations (Kalloo 1992; Warwick 1993; Cole 1994) or development of the alloplasmic lines by transferring the cytoplasm of the wild species to crop brassicas exhibiting

cytoplasmic male sterility (CMS) (Banga 1993). Some of the alloplasmic lines have also exhibited useful agronomic traits (Downey and Rimmer 1993). Chromosome addition and substitution lines were also generated to locate genes on specific chromosomes and for constructing genetic maps. During the last three decades, in vitro techniques such as ovary culture (Inomata 1978; Bajaj et al. 1986; Chevre et al. 1994; Brown et al. 1997), ovule culture (Zenkteller 1990), embryo rescue (Harberd 1969), bridge-cross methods (Rao et al. 1996), and protoplast fusion (Primard et al. 1988) have been used vigorously to obtain a huge number of sexual and somatic hybrids.

10.3 Sexual Hybrids

Conventional breeding methods require a large number of pollinations for obtaining hybrids successfully. In many combinations the success was negligible due to the pre-fertilization barriers (hereafter PRFB) which in most of the cases occur due to the inability of pollen tubes to grow down the style to affect fertilization. A series of observations and experiments in *Brassica* intergeneric and interspecific hybridization, continued through the last three decades, have made it possible to obtain some broader information regarding the mechanism and significance of barriers in wide hybridization. PRFB operate mostly at the level of pollen germination or pollen tube entry into the stigma. In most of the crossability studies, a common feature is that they favor crossability in one direction and that too specifically when the wild species act as female parent. In a few crosses, 34 % of *Raphanus raphanistrum* pollen adhering to the stigma of *Brassica napus* germinated, but no pollen tube penetrated the pistil; however, in the reciprocal crosses, only 12 % of *B. napus* pollen germinated on the stigma of *R. raphanistrum* (Rieger et al. 2001). Cross between *Brassica tournefortii* and *B. rapa* was successful when *B. tournefortii* was used as female parent (Choudhary and Joshi 2001), while it was observed earlier that pollen grains of *B. tournefortii* did not germinate on the stigma of other species resulting in the failure of reciprocal crosses (Harberd 1976). In another investigation, pollen germination was good in those crosses where *Enarthocarpus lyratus*, a wild species, was used as female parent and crop brassicas (*B. campestris*, *B. nigra*, *B. juncea*, *B. napus*, *B. carinata*) as male (Gundimeda et al. 1992). In this study, aniline blue fluorescence of pollinated pistil in intergeneric crosses showed that in many crop brassicas pollen tube grew through the stigma, style, and ovary when *E. lyratus* was the female parent, but in reciprocal crosses, when *E. lyratus* was the pollen parent, very few pollen grains germinated and most pollen tubes failed to enter the stigmatic papillae, few of those which entered the papillae showed swelling of the tube tip and also developed callose plug. None of the pollen tube was observed in the style. In another intergeneric hybridization study between *Diplotaxis siifolia*, a wild species, and crop brassicas, the crop brassicas' pollen germination and pollen tube growth was normal on the stigma of *D. siifolia*; however, in the reciprocal cross, the *D. siifolia* pollen showed strong PRFB; although pollen grains germinated, the

pollen tube failed to enter the stigma. In general, the stigma of the cultivated species inhibits pollen of the wild species, while the stigma of the wild species permits satisfactory pollen germination and the tube growth of the cultivated species (Batra et al. 1989, 1990; Gundimeda et al. 1992; Nanda Kumar and Shivanna 1993). These classical breeding results of direct and reciprocal crosses showed an inherent directional preference (hereafter DP), which ensures these wide hybridizations and overcomes the PRFB.

There are two possible hypothesis to explain the DP, viz., unilateral pollen pistil incompatibility (UI) and endosperm balance number (EBN). The mechanism of UI described in 1955 (Harrison and Darby 1955) explained that the pollen of one species rejects the pistil of another with the reciprocal direction being compatible (De Nettancourt 1977). Most commonly UI occurs with a self-incompatible (SI) species as the pistillate parent and the self-compatible (SC) species donating the pollen. Such a mechanism may be responsible for the reproductive isolation between *B. napus* and the self-incompatible species *R. raphanistrum* (Kercher and Conner 1996). However, some of the wild species including *B. fruticulosa*, *B. maurorum*, *Diplotaxis catholica*, *Erucastrum gallicum*, and *E. cardiminoides* are likely to be more efficient as male parents for developing wide crosses with cultivated *Brassica* species.

Majority of the crosses which do not show PRFB, invariably show postfertilization barriers (PSFB), usually observed as lack of functional endosperm or its early degeneration. The PSFB can be explained by many mechanisms like negative interaction between diverged sequences, global genome rearrangements, widespread epigenetic reprogramming, and imbalance of paternally and maternally imprinted genes in the endosperm. The effects of PSFB are embryo abortion at early globular stage, and lack of functional endosperm (Nanda Kumar and Shivanna 1990). There are many models to explain the operation of PSFB. Dobzhansky–Muller model explains that there is a negative interaction between the genes of two different crossing species, which leads to inviability or sterility in the hybrid offspring (reviewed by Coyne and Orr 1998; Rieseberg and Carney 1998). Another important model is "genomic shock" (allelic incongruity) that causes extensive preprogrammed changes to genomic structure namely, changes in chromosomal organization and repetitive sequences (McClintock 1984).

In most of the interploidy crosses within and between the species, endosperm breakdown is observed as the primary reason for failure of seed development (Watkins 1932; Brink and Cooper 1947; Stebbins 1958; Haig and Westoby 1991). During seed development three different tissues are in intimate contact, viz., embryo, endosperm, and the surrounding somatic tissue of the mother plant. In normal condition the relation between these tissues is 2:3:2. If the uniting gametes have different chromosome numbers, this relation would be altered which would result in poor seed development or complete abortion of seeds (Sikka 1940). Recently the imprinted genes in the endosperm are one of the viable reasons for the PSFB. Endosperm disruption depends upon 2m:1p (maternal:paternal) which is the EBN mentioned above. If maternal genome is in excess (a ratio of >2m:1p), endosperm proliferation inhibits, and if paternal genome is excess (a ratio of <2m:1p), it results in endosperm proliferation (Haig and Westoby 1991; Scott et al. 1998). The mechanism

controlling the parental genomic ratio in the endosperm is called parental imprinting. Parental imprinting is the result of complex theories occurring in endosperm, and one of them is parental conflicting theory. Parental conflicting theory is the fundamental theory which explains the struggle between maternally and paternally derived genomes over resource allocation from mother to offspring (Haig and Westoby 1989, 1991). According to this model the growth promoters essential for endosperm development are expressed when inherited from the father but silenced when they are inherited from the mother, where as growth inhibitors were silenced when inherited from the father and expressed when inherited from the mother. The extra doses of maternal genome provide extra copies of growth inhibitors which lead to small endosperm and seeds. However, extra paternal genome leads to the expression of growth activators which lead to proliferation of endosperm. Both these situations are the result of deviation from normal EBN which leads to an abnormal growth of endosperm (either undergrowth or proliferation). Furthermore, the role of hypomethylation in the parental imprinting was also estimated by antisense *MET1* gene (Adams et al. 2000). These studies were carried out on *Arabidopsis* hybrids, and since *Arabidopsis* is the member of *Brassica*ceae, these studies could be correlated with hybrids of *Brassica*; nevertheless, it is not sufficient to draw a definite conclusion.

In addition to PRFB and PSFB, the genotypes also control the crossability of wide hybrid combinations. The success of interspecific crosses depends not only on the species and direction of cross but also on the genotype and ecotype of species involved in the hybridization (Bozorgipour and Snape 1990). This indeed indicates that a wide range of variation among the different *Brassica* genotypes with respect to their difference in genotypes can lead to difference in pollen fertility in the hybrids. There are several reports on wide crossing which clearly mention about the influence of genotypes and ecotypes on the crossability. For example, the crossability of different *B. napus* cultivars was carried out with *Orychophragmus violaceus*, and it was observed that the crossability of *O. violaceus* was successful with *B. napus* cultivars "Oro," "Huayou No. 8," and "GR 144–149" when *B. napus* was used as female parent. However, no hybrid plants were obtained in the crosses using the cultivars "Canadian twinlow," "Atlex," "81008," and "Senli" (Li et al. 1995). In an another study, two genotypes of *B. tournefortii* ecotypes crossed with the three ecotypes of *B. rapa* i.e *B. rapa* var *trilocularis* (yellow sarsoon), *B. rapa* ssp. *Sarson* (Brown Sarsoon), *B. rapa* var *dichotoma* (toria) showed difference in crossability of different ecotypes (Choudhary and Joshi 2001). It appeared from these studies that genotype in addition with PRFB and PSFB controls the crossability. The presence of strong PRFB and PSFB can be overcome by using tissue culture procedures such as ovary/ovule culture, embryo culture, sequential culture (Inomata 1976; Nanda Kumar et al. 1988a, b; Batra et al. 1990; Gundimeda et al. 1992; Vyas et al. 1990) which involves successive culture of ovaries, ovules, and seeds/embryos and is more effective than simple ovary or ovule culture. Certain other techniques namely grafting, mixed pollination, bud pollination, stump pollination (Hosoda et al. 1963; Sarashima 1964) are also found to be effective in overcoming the barriers.

The first test-tube fertilization in plant was carried out in poppy (Kanta et al. 1962). Test-tube fertilization of excised ovules in *Brassica* is a practical technique for overcoming the PRFB (Kameya and Hinata 1970). The method of in vitro fertilization of ovules can be successfully applied to various species of Brassicaceae. Mature embryos and plants were obtained after in vitro pollination of the ovules of *Arabis caucasica*, *B. napus*, *B. oleracea* var. *sabellica* (kale), *B. oleracea* var. *italica* (broccoli), *Diplotaxis tenuifolia*, *Moricandia arvensis*, and *Sisymbrium loeselii*. In the case of *Sinapis alba*, fertilization and embryo development did not occur. Placental pollination has been successfully used for obtaining hybrid immature embryos at different stages of development from crosses between *B. napus* × *D. tenuifolia*, *B. napus* × *M. arvensis*, *B. oleracea* var. *italica* × *D. tenuifolia*, *D. tenuifolia* × *B. napus*, *D. tenuifolia* × *M. arvensis*, and *D. tenuifolia* × *S. loeselii*. These findings show that in vitro pollination of ovules of various species of *Brassicaceae* makes it possible to perform the whole process of fertilization and embryogenesis and obtain intergeneric hybrid embryos (Zenkteller 1990). Embryo rescue technique is useful as a means for the progress of the study on the interspecific and intergeneric hybridization of crucifer vegetables, where hybrid embryos abort at early stages of development (Nishi et al. 1959; Zhang et al. 2003, 2004; Wen et al. 2008). Mizushima (1950a, b, 1968) carried out the pioneer work of hybridizing species from secondary and tertiary gene pools.

Sexual hybrids show aberrant meiotic chromosome behavior when both the parents are diploid. Chromosome homology between various genomes in *Brassica* has been thoroughly investigated (reviewed in Prakash and Hinata 1980; Prakash et al. 2009 and Prakash 2010). The occurrence of unreduced male as well as female gamete is quite common in *Brassica*ceae (Ripley and Arnision 1990). Cytologically, the hybrids predominant show the presence of univalents, a small proportion of bivalents and even higher associations (tri-, tetra-, pentavalents). The primary reason for the formation of univalents in the wide hybrids is the absence of a homologous partner, relatively less bivalents due to occasional pairing which would bring about low frequency of chiasma formation and ultimate non-conjugation. Bivalents, when they occur, are mostly rod shape monochiasmatic and rarely ring shaped with multiple chiasmata. Multivalents in diploid hybrids occur only rarely. Nevertheless, a variable number of bivalents and frequent trivalents as well as quadrivalents are formed in triploid (tetraploid × diploid) and tetraploid (tetraploid × tetraploid) species combinations.

Wide hybrids have meiotic irregularities such as numerous disjunctional abnormalities including laggards and segregational anomalies (Stebbins 1966) resulting in pollen sterility. The anthers are generally small, flaccid, and empty. The bridge fragment configuration at anaphase I is observed rarely in the wide hybrids, resulted from chiasma formation within a heterozygous inversion. The very rare formation of the bridge occurs, because the length of the inverted segment is so small and does not permit frequent crossover in that region (Sikka 1940).

Among the diploid hybrids, high chromosome pairing has been observed in several combination: *Sinapis arvensis* × *B. nigra* ($2n = 17$, 8II, Mizushima 1952), *Diplotaxis erucoides* × *B. nigra* ($2n = 15$, 6II, Quiros et al. 1988), *B. fruticulosa* × *B. nigra*

(2*n* = 16, 7ii Mizushima 1968), *B. nigra* × *Hirschfeldia incana* (2*n* = 15, III+5II, Quiros et al. 1988), *Erucastrum canariense* × *B. oleracea* (2*n* = 18, 8II, Harberd and McArthur 1980), and *E. cardaminoides* × *B. oleracea* (2*n* = 18 IIV+1III+1II, Mohanty 1996). The triploid and tetraploid hybrids where higher associations have been observed include *B. juncea* × *Diplotaxis virgata* (IIV/2III; Inomata 2003), *B. napus* × *H. incana* (IIV, Kerlan et al. 1993), and *D. viminea* × *B. napus* (2IV, Mohanty 1996). Triploids and tetraploids bivalents and higher associations may result from the homology within the chromosome of the same genome (autosyndesis) or because of intergenomic homology (allosyndesis). In the past, it was difficult to draw conclusions in terms of autosyndesis and allosyndesis. However, it can be stated now with further experimentations that intergeneric homology is always higher than intrageneric homology. Cytogenetics has played an important role in determining the chromosome number, genome analysis, resolving taxonomic status and phylogenetic relationships, genome manipulation, chromosome addition lines for locating genes and introgression, and in situ hybridization for chromosome identification (Chen et al. 2007; Du et al. 2008, 2009). Recent use of genomic in situ hybridization (GISH) (Li et al. 2007; Tu et al. 2009; Ge et al. 2009) and fluorescence in situ hybridization (FISH) enable us to precisely ascertain the degree of autosyndesis and allosyndesis. GISH and FISH assisted in characterization of individual chromosomes, construction of karyotypes, determination of the genomic component of allopolyploid species, analysis of meiotic behavior in hybrids, integration of genetic and physical maps, and studying the genome evolution by FISH mapping. Hybrids between *Brassica* spp and *Orychophragmus violaceus* (2*n* = 24) (an ornamental plant in China) are extensively used to study meiotic and mitotic behavior for introgression of the nuclear genes of interest. All the hybrid combinations were generated when *Brassica* crop species was crossed as female parent with *Orychophragmus violaceus* as the pollen donor (Li et al. 1995, 1996, 1998, 2003; Li and Hensen 1999; Hua and Li 2006; Ge et al. 2006, 2009).

10.4 Somatic Hybrids

Somatic hybridization is an effective technique to overcome barriers to sexual reproduction. Protoplast fusion has been very successful in Brassicaceae (see reviews by Glimelius 1999; Christey 2004; Navarátilova 2004; Li et al. 2005; Fig. 10.4). Biotechnological tools such as embryo rescue and protoplast fusion have made it possible to overcome not only intergeneric but also intertribal incompatibilities. As a consequence 44 somatic hybrids between crop and wild species have been obtained. These represent interspecific/intergeneric [*Brassica spinescens* (Kirti et al. 1991), *Brassica tournefortii* (Stiewe and Robbelen 1994), *Diplotaxis catholica* (Kirti et al. 1995), *Diplotaxis harra* (Begum et al. 1995), *Diplotaxis muralis*, *Eruca sativa* (Fahleson et al. 1997), *Moricandia arvensis*, *Moricandia nitens* (Meng et al. 1999), *Raphanus sativus* (Wang et al. 2006a, b), *Sinapis arvensis*, *Sinapis alba* (Wang et al. 2005), and *Trachystoma ballii*], intersubtribal, and a

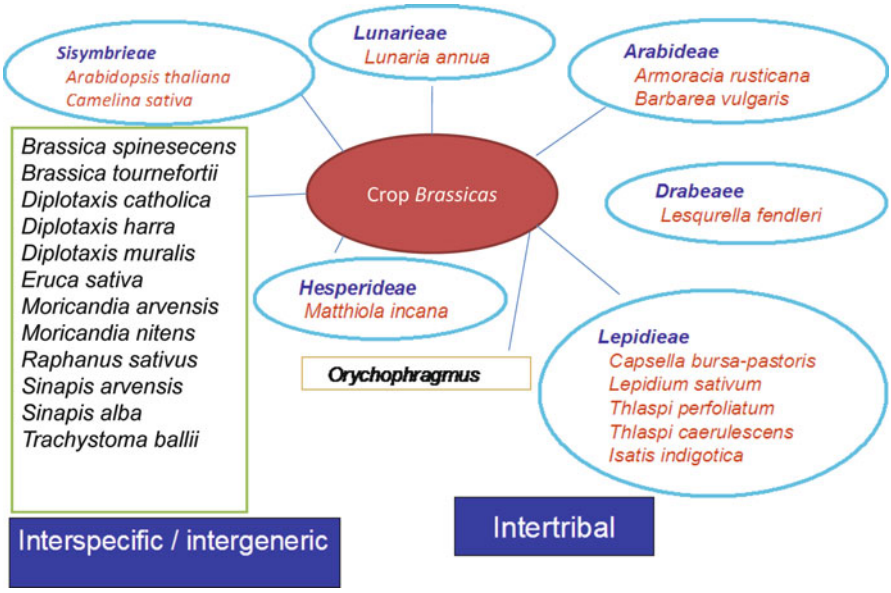

Fig. 10.4 Somatic hybridization involving crop brassicas

substantial number of intertribal combinations from six different tribes, viz., *Sisymbrieae* [*Arabidopsis thaliana* (Siemens and Sacristan 1995), *Camelina sativa* (Narasimhulu et al. 1994)], *Arabidea* [*Armoracia rusticana* (Navarátilova et al. 1997), *Barbarea vulgaris* (Ryschka et al. 1999)], *Drabeaa* [*Lesquerella fendleri* (Nitovskaya et al. 2006)], *Lepidieae* [*Capsella bursa-pastoris* (Nitovskaya and Shakhovskii 1998), *Lepidium sativum*, and *Thlaspi perfoliatum* (Fahleson et al. 1994a, b), *Thlaspi caerulescens,* and *Isatis indigotica* (Du et al. 2009)], *Lunarieae* [*Lunaria annua* (Craig and Millam 1995)], *Hesperiidae* [*Matthiola incana* (Sheng et al. 2008)], and *Orychophragmus* (Li et al. 1995, 1996, 1998, 2003). In many instances, desirable characters have been observed in hybrids. However, the introgression has not been largely possible because of high degree of sterility and lack of sufficient intergenomic chromosome pairing. Nevertheless, the results are not very discouraging and few characters have been incorporated. Examples include somatic hybrids in *Camelina sativa* + *B. carinata* (Narasimhulu et al. 1994) and *Camelina sativa* + *B. oleracea* (Hansen 1998) which were, however, not established as viable field plant due to large phylogenetic distance between the partaking genomes which severely affected the vegetative growth and development of normal plant parts, particularly floral organs. The hybrids are generally intermediate to the respective parents in most of the quantitative characters. Morphologically, they resemble largely the female parent although they also posses distinct male characters, such as 6–8 petals and multiple carpel-like structures in *A. thaliana* + *B. napus* (keller et al. 1993; Bauer-Weston et al. 1993) and 1 or 2 petals in *Thlaspi perfoliatum* + *B. napus* (Fahleson et al. 1994a, b).

A majority of somatic hybrids are seed sterile, if selfed. Generally, with the decrease in the number of alien chromosomes, the fertility increases, and the plants possessing the entire alien chromosome are completely sterile. Somatic hybrids have different possibilities regarding the cytoplasmic genomes; either the parental genomes segregate completely during cell division or both the parental genomes occur as mixed population, and it further leads to recombination in the parental genome (see review by Prakash et al. 2009). The mitochondrial and chloroplast genomes segregate independent of each other. Mitochondrial recombination has been reported to occur frequently in *Brassica* (Glimelius 1999). On the contrary, intergenomic chloroplast recombination is rare. In hybridization involving wild species, generally the chloroplast from the crop parent is favored and the higher the ploidy of the partaking genome is, the more is the contribution of the chloroplast per cell (Butterfass 1989). Mostly, *B. napus* or *B. juncea* allopolyploids take part in hybridization with diploid wild parents; therefore, the chance of crop species chloroplast is more. In comparison to the symmetrical fusion, the asymmetrical fusions (irradiating donor (wild) protoplast to induce double-strand break) are having more chances of survival and adaptability because only a fraction of alien genetic content is present.

10.5 CMS Systems Originated from Wild Taxa

The most rewarding utilization of wild species in crop brassicas has been in synthesizing alloplasmic lines of crop species exhibiting male sterility with the wild species being as cytoplasm donors (*Arabidopsis thaliana*, *B. oxyrrhina*, *B. tournefortii*, *Diplotaxis muralis*, *D. erucoides*, *D. berthautii*, *D. catholica*, *D. siifolia*, *Eruca sativa*, *Erucastrum canariense*, *Enarthocarpus lyratus*, *Moricandia arvensis*, *Orychophragmus*, *Raphanus sativus*, *Sinapis arvensis*, *Trachystoma ballii*) and introgression of male fertility restoration genes. Out of these CMS sources, fertility restoration has been identified in *Raphanus*-based Ogura CMS and Polima CMS in the western countries, and it has been detected in the CMS-based crosses in *B. tournefortii*, *B. juncea* CMS, *Polima* CMS, and *Siifolia* CMS in India (Rai et al. 2007). Heterotic *B. napus* hybrids based on *Raphanus/Ogu* system in Europe and Canada and *B. juncea* hybrids on *Moricandia arvensis* (Prakash et al. 1998) system in India have been developed.

10.6 Monosomic and Disomic Addition Lines

Wild germplasm needs to be thoroughly characterized for different traits. Dissecting their genomes and developing chromosome addition lines to locate gene(s) of importance will accelerate the map-based cloning of these genes. Monosomic addition

lines are used for dissecting several *Brassica* and related genomes, viz., *B. nigra*, *B. oleracea*, *B. rapa*, *B. oxyrrhina*, *Diplotaxis erucoides*, *Raphanus sativus*, *Sinapis alba*, *S. arvensis*; and several disomic addition lines have been developed including *A. thaliana–B. napus* (Leino et al. 2004), *B. napus–S. alba* (Wang et al. 2005), and *B. napus–C. abyssinica* (Wang et al. 2006a, b). A full set of nine disomic *B. napus–R. sativus* addition lines developed by Budahn et al. (2008) is the first disomic addition line series in Brassicaceae.

10.7 Introgression of Nuclear Genes from Wild *Brassica* Species for Breeding

Nuclear genes for abiotic and biotic stress tolerance have been successfully introgressed in the crop species to generate progenies of wide hybrids with additional useful traits for breeding. The agronomic characters in wild species are an attraction to breeders including resistance to various biotic stresses such as beet cyst nematode, alternaria blight [*Diplotaxis erucoides* (Klewer et al. 2003), *Camelina sativa*, *Capsella bursa-pastoris*+*B. napus*, *Coincya* spp.], blackleg [*Arabidopsis thaliana*+*B. napus* (Saal et al. 2004), *Sinapis arvensis*)], flea beetle (*Crambe abyssinica*), and other traits such as C3–C4 intermediate photosynthesis [*Moricandia* spp. (Bang et al. 2003), *Diplotaxis tenuifolia*]; drought tolerance (*Brassica tournefortii*, *Diplotaxis acris*, *Eruca* spp., *Lesquerella* spp.); cold tolerance (*Coincya richeri*, *Erucastrum abyssinicum*); high erucic acid content [*Barbarea* spp., *Cardamine*, *Lepidium* (Hu et al. 2002)]; increased level of palmitic and linolenic acids from *Orychophragmus* (Wang et al. 2003); greater amount of erucic acid from *Crambe abyssinica*+*B. napus* (Schroder-Pontoppidan et al. 1999); high amount of lesquerolic acid from *Lesquerella fendleri*+*B. napus*; fertility restoration from *R. sativus Diplotaxis catholica Moricandia*, *Trachystoma balli*, and *Sinapis arvensis* (see review Prakash et al. 2009); zinc and cadmium accumulation (*Thlaspi caerulescens*+*B. napus*); and high nervonic acid content (Fahleson et al. 1994a, b, *Thlaspi perfoliatum*+*B. napus*). Breeding for improvement of fatty acid composition in rapeseed has been emphasized for high-quality rapeseed breeding. Most of the Asian varieties have high oil content, but lower oleic and higher erucic acid content (Fig. 10.5) than the European varieties. The variation in the oleic and erucic acid content in the European varieties were larger than in the Asian varieties. *Brassica-related* wild germplasm will have an increasingly important role to develop abiotic and biotic stress free and better adapted cultivars in the future. Their utilization will be more successful using cellular and molecular biotechnological tools.

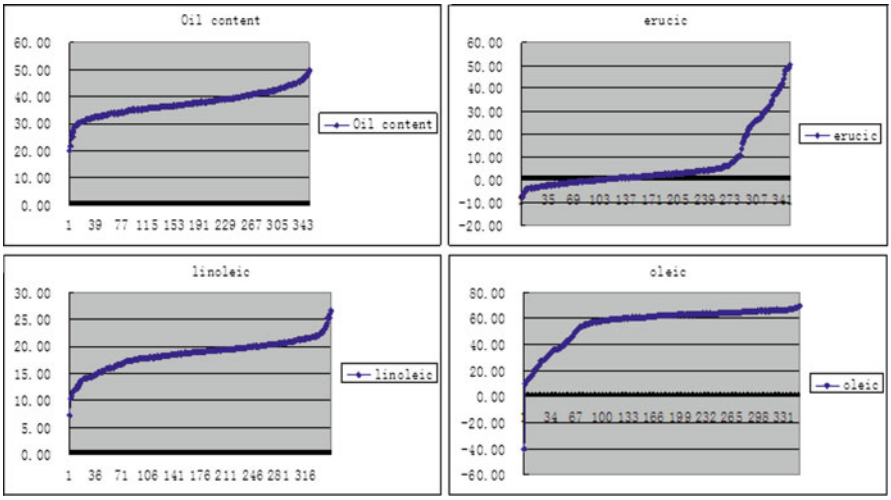

Fig. 10.5 Useful traits incorporated in *Brassica* crops for breeding

10.8 Epigenetics of Interspecific and Intergeneric Hybrids: Nucleolar Dominance

In distant hybrids, it is often observed that the ribosomal genes of one species (one genus) are transcriptionally dominant over the ribosomal genes of other species (other genus). This phenomenon is known as Nucleolar Dominance (hereafter ND) (see review by Pikaard 1999, 2000, 2001). It occurs both in the hybrids of animal and plant kingdom like *Xenopus*, *Drosophila*, *Crepis*, *Salix*, *Ribes*, *Solanum*, *Hordeum*, *Avena*, *Agropyron*, *Triticum*, Zea, *Triticale*, *Brassica*, and mammalian cells. It is an epigenetic effect and is second only to inactivation of one X chromosome that occurs in the somatic cells of female mammals. However, unlike X inactivation, the choice of which set of rRNA genes to silence is not random, rather there is dominant and underdominant rDNA, playing a crucial cross talk in ND (wheat–rye addition line). ND is independent of maternal and paternal imprint. ND studies on plants is having a history of more than seven decades, and it was for the first time described by M. Navashin, a Russian cytogenetist, who began a series of caryological investigation on the plant genus *Crepis*. In 1928, he found that there is a reversible change in the chromosome morphology. Navashin used the term "amphiplasty" to describe the ability of metaphase chromosome to adapt new forms. Navashin stated that "It was a great surprise to find that the chromosomes of two or more different species brought together by hybridization in certain specific combination suffer striking alterations of their individuality (Navashin 1934)." In his study out of 21 different hybrid combinations, eight hybrid combinations have both the D chromosome (earlier the chromosome were designated with alphabets) of the parent at metaphase. In other 13 hybrid combinations, one progenitor had retracted its

satellite. NORs include active rRNA genes which give rise to secondary constriction of metaphase chromosomes and silent rRNA genes which are often highly compacted in dense heterochromatin. Each rRNA genes at NOR is nearly identical in sequence. Differences in the number of repeated DNA elements occur commonly in the intergenic spacer region. These unparalleled investigations done by pioneer researcher have paved the way for the study of this amphiplastic (ND) phenomenon. Hierarchy of rRNA gene transcriptional dominance in *Brassica* is *B. nigra*, BB>*B. rapa*, AA>*B. oleraceae*, CC (Chen et al. 1997) (Fig. 10.1). So, the size of repetitive region and the number of repetitive elements of NORs are not the determining factors for ND in crop *Brassica*. In the last decade a lot of studies were carried out on epigenetics, and it was emphasized that the ND is an epigenetic phenomenon, and DNA methylation and histone modification play a crucial role in maintenance of nucleolar dominance in allotetraploids of *Brassica*. Hypermethylation and hypoacetylation lead to transcriptional silencing of rDNA of one of the parents in the hybrid. A role of methylation and acetylation was dramatically revealed in allotetraploid *Brassica* by using inhibitors of cytosine methylation (like 5-aza 2′-deoxycytidine) and histone deacetylase (HDAC) inhibitors like sodium butyrate and trichostatin A (TSA).

10.9 Conservation of Wild Germplasm

Collection and conservation of all the different wild relatives, species, and relative of the cultivated species (followed by the evolution of their characteristics) are a prerequisite for the effective exploitation of the natural genes available in the populations. During the 1970s wild germplasm of *Brassica* were extensively collected and cytogenetic studies were started. In many crops preexisting genetic variability is available from wild relatives of crops. The vast collection of wild crucifers was carried out by the expeditions to the Mediterranean region (1970–1975) by Spanish researcher Prof. Cesar Gómez-Campo and Japanese researchers U. Mizushima, S. Tsunoda, and K. Hinata. Mizushima initiated investigations on wild germplasm in the early 1970s, executed hybridizations between wild and crop species, and studied observations on chromosome pairing and interpreted genome homoeology (Gómez-Campo and Gustafsson 1991). Harberd (1972, 1976) classified germplasm referred to *Brassica* coenospecies into cytodemes (crossing groups) and studied the chromosome pairing in a large number of interspecific and intergeneric hybrids. Intensive efforts have been made in the last decade to search and collect this material; otherwise, it would have been invariably lost. Most of the *Brassica* collections are conserved by means of seeds, and in general, they are conserved under long-term storage condition to maintain seed variability for many years. The only exception within the *Brassica* crop is perennial Kale that is vegetatively propagated (Gómez-Campo 1999a, b). Ex situ conservation of plant genetic resources in gene bank involves collecting traditional varieties and landraces from around the world and, in particular, from centers of genetic diversity of specific crops. The ex situ conservation also involves conservation and maintenance of these accessions for current and future users for regeneration activities.

Climate, plant diseases, insect pests, and market demands for new quality traits are included in the broad definition of environment. Wide hybridization is an important tool to introduce alien variation into the cultivated crops. Alien gene transfer has played an important role in creating additional genetic variability in crop species; introgression of newer, useful, and desirable alleles; and devising several innovative and advanced techniques like preferential chromosome elimination leading to techniques like doubled-haploidy breeding. Somaclonal variation during the culture phase of hybrid embryos developed through distant hybridization generates additional avenues of variability in several crop plants. The impact of alien gene introgression has been well seen during the "Green Revolution" and also after that in the development of improved plant varieties in a spectrum of crops including cereals, pulses, oilseeds, and vegetables and ornamental and horticultural crops. Wild species are a rich reservoir of several useful alien genes which are no longer available within the cultivated gene pools. Continuous efforts have been underway to collect and conserve wild relatives of various crops in national and international gene banks and use them for alien gene transfer into the cultivated background.

10.10 Conclusions and Future Prospects

Distant hybridizations in crop brassicas through sexual cross can be traced back to the 1950s and through somatic hybridization to the late 1970s. The sexual hybrids and their progenies recovered by the aid of special efforts such as repeated pollination, embryo rescue, and sequential culture have contributed immensely in widening the genetic base of cultivated brassicas as well as generating newer genetic variability providing additional avenues of selection. This has resulted in the transfer of several desirable genes into cultivated background from wild species including those for disease and insect-pest resistance, improved oil quality, fatty acid composition, male sterility and fertility restoration systems, and, of late, tolerance to a few abiotic stresses. Nevertheless, pre- and postfertilization barriers in producing viable hybrids and their progenies have slowed down the progress in transferring useful alien genes. In such case somatic hybridization has played a great role, and among all crop plants, *Brassica* has been one of the most cited examples where somatic hybridization has witnessed great success.

The vast knowledge of molecular markers has also greatly aided in *Brassica* improvement, and it has helped in mapping important traits. The sequencing of *Arabidopsis* genome has proven to be of great use in marker-assisted breeding. This knowledge further needs to be integrated with conventional breeding or transferring alien genes through AB-QTL approach. Further strides are required in genetic transformation and intragenesis and cisgenesis to deploy alien genes from across the genome boundaries for the genetic improvement of crop brassicas. At the same time, further improvements are required in somatic hybridization and tissue culture protocols, especially to solve the problems of low regeneration rate and genotype dependency. For a direct use of wild species in genetic improvement of crop brassicas, chromosome fragmentation and integration of only a specific fragment

conferring a useful trait will be a more practical approach in improving *Brassica* utilizing wild species.

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Chapter 11
Oil Palm and Coconut

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Abstract Coconut and oil palm are the two important plantation crops grown in the wet tropics. Both the palms are monocots and produce oil from their fruits. Oil palm currently occupies the topmost position in the international vegetable oil market, while coconut is a palm with diverse uses in addition to being an oil crop. Out of the two palms, oil palm has shown a spectacular boom in the world oil trade during the last 60 years. Systematic genetic improvement programmes of both these palms have been carried out since the first quarter of the last century due to the importance of oil palm and coconut. Oil palm and coconut share much similar challenges in their genetic improvement for better traits owing both of these being perennial monocot palms of massive stature, cross-pollinating heterogeneous populations and certain other genetic implications. Despite these inherent constraints, considerable gains have been achieved in the breeding of both oil palm and coconut through the transfer of desirable genes from diverse sources into the cultivated material. African oil palm populations have been most important in transfer of genes from populations of different origins. In addition, interspecific hybridization between African and American oil palms has also been carried out with success. Similarly, in coconut, much advancement has been made with respect to yield components, precocity and shorter stature using intervarietal and interpopulation gene transfer methods. These gene transfer methods have helped to bring oil palm to the topmost position in the world oil trade and coconut to provide vegetable oil and livelihoods to millions of people in the coconut-growing countries.

Keywords Coconut • Intervarietal hybridization • Interspecies hybridization • Oil palm • Wide crosses

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11.1 Introduction

Oil palm and coconut are two of the most important tropical oil crops. Both these plants are monocots belonging to the family Arecaceae and produce vegetable oil from their fruits. Oil palm has been the most important oil crop in the world surpassing soybean since 2006. Coconut oil, although produced in much less quantities, continues to be the main cooking oil in many of the coconut-growing countries in the world. Programmes for the genetic improvement of the two palms have been in place during the last several decades due to the importance of these crops in many tropical countries. Oil palm and coconut share much similar challenges in their genetic improvement for better traits owing to both of these being perennial monocot palms of huge stature, cross-pollinating heterogeneous populations and a number of other genetic implications. Despite these inherent constraints, considerable gains have been achieved in breeding of both, oil palm and coconut, through the transfer of desirable genes from diverse sources into the cultivated material. As a result, both oil palm and coconut have managed to maintain their respective positions in the world trade of oil and livelihoods of people depending on these palms.

11.2 Oil Palm

Oil palm cultivation first began in Africa where it still makes an important contribution in the diets of people and as an industrial raw material. However, oil palm made an economic revolution in the Southeast Asia, especially in Malaysia and Indonesia, with Thailand and Papua New Guinea also accommodating significant plantations. Total cultivated area in Malaysia increased from 300,000 ha in 1970 to over 4.0 million ha in 2008. Total area under oil palm cultivation in Indonesia was over 5.0 million ha in 2008 giving evidence for the economic importance of this crop in these countries and indicating the increase in the world demand for palm oil. Meanwhile the production in the African countries has also begun to increase, and plantations have been started in the South American countries also (Corley and Tinker 2003).

Among all the oil-producing plants in the world, oil palm produces the highest oil content per hectare per year recording 12 t/ha in some experimental plots and averaging between 3 and 7 t/ha in commercial cultivations. With this high productivity, palm oil contributes to about one-fourth of the total world production of fats and oils (Oil World Annual Report 2010). Two types of oils are extracted from the fruits of oil palm. Palm oil or internationally known as crude palm oil (CPO) is extracted from the mesocarp of the fruit, while palm kernel oil (PKO) is extracted from the kernel of the fruit. CPO is the predominant oil out of the two, with a production of 95 % of the total palm oil production. Palm oil is used in several different ways, mainly as a cooking oil and margarine, vegetable ghee and shortening production, while about 10 % of the palm oil produced is used for industrial purposes as oleo chemicals, cosmetics and more recently as biofuels also. Biodegradable plastics, pharmaceuticals and nutraceuticals are the recent products demanding palm oil.

11.2.1 Origin

The oil palm has been hypothesized to originate in Gondwanaland which disappeared in the drift of American and African continents in prehistoric era (Zeven 1965) resulting in the separate evolution of African oil palm (*Elaeis guineensis* Jacq.) and American oil palm (*E. oleifera*, previously *E. melanoccoca*).

There are many historical (Opsomer 1956; Surre and Ziller 1963; Zeven 1965) and fossil (Raymond 1961; Zeven 1964; Ergo 1997; Sowunmi 1999) evidences for the evolution of the African oil palm along the gulf of Guinea. There are wild and semiwild oil palm groves along the coastal belt starting from northernmost Senegal through Sierra Leone, Liberia, Ivory Coast, Ghana, Togo, Benin, Nigeria, Cameroon and Angola up to the southernmost Democratic Republic of Congo (Ngando-Ebongue et al. 2010). Much of the diversity of African oil palm is believed to be centred in the tropical forests of Nigeria, Cameroon, Congo and Angola. The oil palm cultivation in Southeast Asia was started in 1848 with four seedlings derived from the same fruit bunch of a palm in West Africa and planted in Bogor Botanic Gardens in Java, Indonesia. The entire Southeast Asian palm groves are believed to descend from these four palms. The American oil palm is endemic to the tropical region of Central and South America, starting from Mexico in the north to the Atlantic coast with a discontinuous distribution.

11.2.2 Biology/Botany

Oil palm belongs to the family Arecaceae and the genus Elaeis. The genus *Elaeis* includes two species, viz., *Elaeis guineensis* (Jacq) (African oil palm) and Elaeis *oleifera* (previously *E. melanoccoca*) (American oil palm). Oil palm is a diploid with a chromosome number of $2n = 32$ (Maria et al. 1995) with a genome of 1C = 980 Mbp (Bennett and Smith 1991; Bennett and Leitch 2005), as discovered from the studies on African oil palm. The plants are monoecious producing distinct male and female inflorescences in different cycles. It is mainly outcrossing with the varying lengths of male and female cycles largely depending on the genotype and the environment (Corley and Donough 1995).

11.2.2.1 *Elaeis guineensis*

The African oil palm *E. guineensis* is the most widely planted commercial species. This perennial palm displays an indeterminate growth of its cylindrical pseudostem with a large crown consisting of 30–45 green leaves. After reaching the reproductive stage around 2–3 years after field planting, an inflorescence is initiated in the axil of every leaf as compact and ovoid masses having many spines. Palms usually produce 1–2 inflorescences per month, and these can be female or male depending on whether the palm is in male or the female phase (Adam et al. 2005). The female

inflorescence, upon pollination, develops into a fruit bunch in about 22–26 weeks after pollination (Ngando-Ebongue et al. 2010). The weight of fruit bunches varies from about 10–50 kg having a total of about 500–4,000 fruits/bunch averaging about 1,500. The oil palm fruit is a sessile drupe of generally ovoid shape weighing about 3–30 g. The fruit turns red to brown at maturity and consists of the pulp which is the mesocarp, shell and the kernel. The mesocarp is about 60–90 % of the fruit weight and 35–55 % of the bunch weight and produces edible, orange-reddish-coloured oil which is referred to as palm oil. The kernel or the endosperm produces a clear yellowish colour oil, which is known as palm kernel oil, and this oil is similar to coconut oil. In African oil palm thickness of the shell is a main characteristic, because it is the criterion which distinguishes the three types of palms within this species. Shell thickness is a trait, which shows monogenic inheritance of the shell gene (*Sh*). The three types of oil palm populations are named Dura, Pisifera and Tenera out of which Dura is the predominant type with a frequency of about 97 % in the wild palm groves (Ngando-Ebongue et al. 2010). Dura palm is characterized by a thick shell of about 2–8 mm in thickness and a mesocarp to fruit percentage of 35–75 %.

The shell is not present in the fruit of Pisifera which is the second type of *E. guineensis* palm. This type contains a high mesocarp to a fruit ratio of 90–99 %. Pisifera palms are rare in the wild, and it is characterized by a higher degree of female sterility due to the common characteristic of dying of female inflorescences before reaching maturity.

The third palm type is termed Tenera, and it contains a thin shell in the range of 0.5–2 mm and a thick fibrous mesocarp. The mesocarp to fruit ratio varies between 55 and 96 %. Tenera form resulted from a cross between Dura and Pisifera palms. Tenera hybrids, produced by artificial pollination, have been widely used in commercial plantations worldwide.

11.2.2.2 *Elaeis oleifera*

American oil palm *E. oleifera* has been evolved in the Central and South America (Rajanaidu 1986). *E. oleifera* is characterized by a slow-growing shorter stem reaching to about one-fifth of the height of *E. guineensis*. This palm also often becomes procumbent with an erect crown when it reaches maturity (Hartley 1988). The leaflets of *E. oleifera* form only in one plane along the leaf petiole similar to coconut, while in *E. guineensis*, more numbers of leaflets originate from several planes. *E. oleifera* also produces distinct male and female fruit bunches, while the pollen is of foul smell. The fruit bunches are of conical shape, spiked with shorter spines and they are partly covered by spathes even at maturity. They bear smaller fruit bunches than the African oil palm with an average weight of 8–12 kg, and the fruits are also smaller with a shell thickness of 1–3 mm. The mesocarp to fruit ratio is between 29 and 50 % for normal fruit amounting up to 80 % in parthenocarpic fruit (Corley and Tinker 2003). The fruits, at maturity, turn orange in colour. The palm oil extracted from the mesocarp of *E. oleifera* fruits contains a high unsaturated fatty acid content (59–90 %) as compared with *E. guineensis. E. oleifera*, the American oil palm, is inferior with respect to bunch yield and oil extraction rate when compared to *E. guineensis*.

Table 11.1 Genetic resources of oil palm and their important characteristics

Species	Country	Important characteristics
E. guineensis	Nigeria	Higher unsaturated fatty acid Content, short stature
E. guineensis	Zaire and Cameroon	Tolerance to fungal disease Ganoderma
E. guineensis	Angola	Larger fruits with high carotene content
E. oleifera	Ecuador	Thick mesocarp, small stature
E. oleifera	Costa Rica	High oil yield
E. oleifera	Suriname	Compact stature
E. oleifera	Colombia	High unsaturated fatty acids percentage

11.2.3 Goals in Oil Palm Breeding

Yield increment is the most important and the commonest goal in breeding any crop; this holds true for oil palm also. Yield in oil palm consists of two components: bunch yield (number of bunches and bunch weight) and oil content per bunch (number of fruits/bunch and ratios of mesocarp/fruit and oil/mesocarp). In addition to yield, there are several other oil palm improvement objectives which may vary from country to country depending on their relative importance. For example, dwarf stature is an important objective in countries like Malaysia where labour is a limitation for harvesting tall palms. In addition to these, high oleic acid content, resistance to diseases such as Ganoderma (mainly in Southeast Asia), *Fusarium* wilt resistance (in Africa), bud rot and fatal yellowing (in South and Latin America) and drought tolerance (mainly in Africa) are among the major goals in oil palm improvement programmes.

Certain genetic resources of oil palm have been reported to carry genes which are essential to achieve the above objectives (Soh et al. 2010). Table 11.1 summarizes some of the traits reported in different oil palm populations.

11.2.4 Genetics and Breeding of Oil Palm

There are two factors dominating oil palm breeding research, namely, shell thickness of the fruit and the long selection cycles. Shell (endocarp) thickness is controlled by a major gene *Sh* (Beinaret and Vanderweyen 1941). Dura palms are homozygous for the thick shell, while the Pisifera palms are the other homozygotes producing no shell. The heterozygote, Tenera, is thin shelled. The lack of a shell in Pisifera is proposed to be caused by a mutation in the *Sh* gene resulting in failure of lignification of the region where the shell would form (Sparnaagi 1969; Bhasker and Mohankumar 2001). In Dura, wild gene homozygous alleles replace 30 % of the mesocarp with shell thereby reducing the oil yield derived from the mesocarp. Pisifera, although lacking in a shell, does not give increased yields because in most of the Pisifera germ plasm, fruit bunches abort during development resulting in negligible yield. Certain female fertile Pisifera germ plasm has also been identified, suggesting a close linkage of the shell thickness *Sh* gene with a fertility gene.

Selection cycles in oil palm are long. For Dura female plants, it is about 10–12 years, while for Pisifera male parents, the selection cycles are of about 16 years. This indicates a limited number of selection cycles over the oil palm improvement programmes compared to many of the shorter duration plants.

Several breeding methods have been adopted in oil palm with due considerations to the cross-pollinating breeding behaviour of oil palm. Out of these, reciprocal recurrent selection and the family and individual selection are the two most widely used breeding methods. In addition to these two breeding methods, backcross breeding has been used for the introgression of desirable characters from unimproved genotypes to a commercially viable genetic background. Furthermore, breeding methods for clonal propagation and index and BLUP selection (Soh 1994) have also been attempted in oil palm breeding over the years.

11.2.5 Alien Gene Transfer in Oil Palm

Many of the above-mentioned breeding methods in oil palm are concerned with transferring a trait of importance from one genetic background to another. This transfer of novel genes to a different genetic background is called the alien gene transfer in the sense that the transferred gene, or in most cases the allele, has previously been lacking in the recipient genotypes. Alien gene transfer in oil palm in most occasions has been between populations of different origins. However, more distant gene transfers, i.e. interspecific hybridizations, have also been successfully achieved in this perennial oil crop.

Transfer of genes essentially requires genetic materials which differ for the trait that is transferred. As discussed previously, there are two species in the oil palm genus Elaeis. However, the majority of the gene transfer in oil palm has been between the populations of different origins. In oil palm genetic resources, there are no varieties as per, the exact meaning of the word variety (Soh 1999). However, Dura, Pisifera and Tenera are the three main types of oil palm which have formed the base material for the genetic improvement of this palm. In addition to these palm types, populations of different origins of these genetic resources have been observed to have considerable differences among them. These populations have widened the genetic base of the oil palm breeding material over the last few decades.

11.2.6 Genetic Resources of Oil Palm for Alien Gene Transfer

Oil palm was introduced to Malaysia and Indonesia by the European colonists. Four Dura seedlings known to have obtained from the same fruit bunch of a palm in West Africa and planted in Bogor Botanic Gardens in Indonesia formed the base material for the plantations in these two countries. The progenies resulted from selections and hybridizations among these thick-shelled Dura plants were planted in Deli

Province in Sumatra and from there were taken to Malaysia (Rosenquist 1986). This population was named as "Deli Dura" and became a commercial cultivar in both Malaysia and Indonesia from 1911 to the 1960s. Such populations which are derived from a few progenitors resulting in a narrow genetic base are referred to as Breeding Populations of Restricted Origins (BPRO's) (Rosenquist 1986).

Deli Dura BPRO has given rise to several subpopulations by distribution to different localities and subsequent selections. La Me Deli, Ulu Remis Deli, Serdang Deli and Elmina Deli are examples for such Deli populations. The two populations Dumpy and Gunung Melayu are dwarf type mutants of Deli BPRO.

AVROS BPRO is a Pisifera population. It has been derived from a high-performing single plant named Djongo located in Zaire and was developed in Sumatra. AVROS BPRO has been widely used in oil palm breeding programmes in Malaysia, Indonesia and Papua New Guinea, Colombia, Costa Rica and Thailand. Characteristic features of AVROS BPRO are vigorous trunk growth and large fruits with thick mesocarp producing high yield.

Yangambi BPRO was produced in Congo at Yangambi Research Station from open pollinated progenies of the Djongo palm and other Tenera palms from Yawenda, Isangi and N'gazi. Main characteristics of Yangambi BPRO are similar to those of AVROS BPRO. However, a short-stemmed Yangambi variant also has been developed in this BPRO.

- La Me BPRO was developed in Ivory Coast by CIRAD (French Agricultural Research Centre for International Development), which was then known as IRHO. The ancestral seeds of this Tenera population have been collected from the wild oil palm groves in the Ivory Coast. Smaller stature, high number of smaller bunches bearing smaller fruits and the tolerance to less favourable growing conditions are the characteristics of this BPRO. La Me BPRO is a base material in West Africa and Indonesia oil palm breeding programmes guided by CIRAD.
- Ekona BPRO has been developed from the wild palms in the Ekona area in Cameroon by the Unilever plantation group. Certain progenies of this BPRO are high yielding and show resistance to Fusarium wilt disease. This BPRO generally bears smaller fruits with high oil content. Ekona BPRO has been distributed to Malaysia, Costa Rica and Thailand through Unilever group.
- Calabar is a BPRO developed in Nigeria by the Nigerian Institute for Oil Palm Research (NIFOR). Progenies of NIFOR's Calabar selections have been distributed to Ghana, Costa Rica, Indonesia and Malaysia also (Soh et al. 2010).

11.2.7 Derived and Recombinant BPROs

BPROs are interbred or introgressed to develop new BPROs from the traditional BPROs. The main objective is to transfer the desirable genes from traditional BPROs to new recombinants to have mixed favourable genes in the resultant progenies. Ulu Remis teneras, Dumpy AVROS, Dumpy.Yangambi.AVROS, La Me × Dumpy AVROS are some examples to derived and recombinant BPROs (Soh et al. 2006).

11.2.8 Achievements and Impacts of Alien Gene Transfer in Oil Palm

Oil palm yields have recorded a considerable increase over the last 5–6 decades. Out of the total increase, 70 % is reported to be due to genetic improvement and 30 % to the improvements in agronomic practices (Davidson 1993). A major portion of the yield increment caused by breeding is due to the transfer of genes from Pisifera parents into Dura parents to produce Tenera hybrids.

11.2.9 Tenera Hybrid Improvement

Dura (D) type Deli populations were the only commercial planting material in Malaysia and Indonesia until the 1960s. With the elucidation of the monogenic inheritance of the shell gene (*Sh*), the cross between the thick-shelled (D) parents with the shell-less Pisifera (P) parents was observed to result in 100 % thin-shelled Tenera (T) palm, indicating incomplete dominance of the shell gene. D palms were used as the female parent while P palms brought to Southeast Asian region from Africa always served as the male parent in T development owing to the common female sterility of the P palms. The transfer of the wild allele of the gene *Sh* from P populations to the cultivated type D is the first reported transfer of alien gene between the populations of oil palm.

The evaluation of early Tenera hybrids revealed the presence of important differences with respect to vegetative characters, bunch yield components and the ratio of oil to mesocarp between the palms from different origins. Based on these results, two groups of *E. guineensis* were identified. The palm in group A is characterized by a small number of large bunches (Southeast Asian Deli palms), while group B palms produce a large number of smaller bunches, and these are generally from Africa. The early studies further revealed the significantly higher yielding capacity of crosses between Asian Deli and African populations. This inter origin gene transfer led to the success of Tenera hybrids (Gascon and de Berchoux 1964). The findings also led to the choice of parents from between the different origins; group A Deli as female parents and group B, African Pisifera palms, as male parents.

Upon the introduction of T hybrid, it soon became the most favoured planting material for commercial plantations of oil palm in a very short period of time (Hartley 1988). The switchover of planting material from the thick-shelled thinner mesocarp (60 % mesocarp to fruit content) producing D palms to thin-shelled, thicker mesocarp producing T (80 % mesocarp to fruit content) would account for a minimum of 30 % mesocarp oil yield increase in addition to the increase in the yields of FFB (Soh et al. 2009). During a period of two decades, about two generations of improved T materials have been planted, reporting an increase of oil yield from 5 to about 10 tons/ha/year in research trials. Lee et al. (1990) and Rajanaidu et al. (1990) estimated a T improvement of 6–7 % by progeny testing the same Ps on

Fig. 11.1 A Tenera hybrid oil palm

two successive generations of selected Ds. Selecting the best 15 % of P in hybridization resulted in a 12 % yield increase (Lee and Yeow 1985). However, in commercial seed production, about 30–50 % of the top P parents are used resulting in a yield increment of only 10–15 % per generation for the T hybrids, which is reasonable with the selection of both the parental populations (Soh et al. 2003). Therefore, the average yield increment of 15 % per generation over two successive generations estimated by Hardon et al. (1987) is due to the T hybrid improvement with selection of both the parental populations (see Fig. 11.1).

11.2.10 Interspecific Hybrids in Oil Palm

Much of the breeding and genetic improvement efforts in oil palm were centred on the species *E. guineensis*, the African oil palm. The American oil palm species *E. oleifera* received much less attention up until recently, due to its low bunch yield and poor oil extraction rate compared to its African relative. However, *E. oleifera* possesses its own desirable traits which have been left unexploited until recently in commercial plantations. Oil extracted from the mesocarp of the fruits of *E. oleifera* palms are characterized by a high saturated fatty acid content of 59–91 % as against 25–72 % for that of *E. guineensis* (Ngando-Ebongue et al. 2010). In addition, shorter stem, compact growth habit and, most importantly, the resistance to fatal yellowing are the economically important characteristics of *E. oleifera*.

Transfer of genes for fatal yellowing, shorter stature and changes in fatty acid composition from *E. oleifera* to *E. guineensis* planting material has been given priority in the breeding programmes in several countries. To achieve this gene transfer, the interspecific hybrid, *E. oleifera* × *E. guineensis*, has been attempted. Despite being of two different species and geographical isolation, the two species were found to be cross compatible, and fertile hybrids could easily be obtained (Hardon 1969; Hardon and Tan 1969; Amblard et al. 1995). However, the major constraint in this effort was the poor fertility of the hybrids resulting sometimes in seed abortion (Viegas and Muller 2000). Also on certain occasions, fruit set in interspecific hybrid was found to be poor (Hardon 1969; Meunier and Boutin 1975). These problems however were not common in all the attempts at producing and planting of this interspecific hybrid. Countries plagued with fatal yellowing do not have the option of planting *E. guineensis*, and therefore, moving forward with the interspecific hybrid was essential. The fertility problem of this hybrid was observed to be worse with the *E. oleifera* material from Central American origin. *E. oleifera* germplasm has been categorized based on geographical location as Central American, Brazilian and Surinamese. These different germplasm have been observed to produce different hybrids with *E. guineensis*, although remarkable differences have also been observed between progenies from the same origin (Meunier et al. 1976).

The oleifera material used in the hybridization programme has not undergone much selection process compared to the stringent selection and improvement programme of the *E. guineensis* material. Moreover, the germplasm of *E. oleifera* has neither been conserved nor been characterized to the extent of *E. guineensis* (Rajanaidu et al. 1989). Consequently, prospects for the *E .oleifera* × *E. guineensis* hybrid are not gloomy if efforts are made to properly collect, conserve, characterize and utilize the *E. oleifera* germplasm followed by selection of parents prior to hybridization.

E. oleifera × *E. guineensis* interspecific hybrid, although is not competitive with commercial *E. guineensis* with respect to crude palm oil production, provides the only option of planting material in the fatal yellowing-affected areas such as in the American region. The primary objective of the oil palm industry in these areas is to supply the local market with refined palm oil, and for this purpose, the interspecific hybrid is sufficiently competitive mainly due to its observed resistance/tolerance. There are other uses of this hybrid in addition to the oil which is rich in unsaturated fatty acids (Rajanaidu et al. 1989). The height increment of *E. oleifera* × *E. guineensis* hybrid is much less than the commercially preferred cultivars, increasing the economic lifespan up to about three times, depending on the combination of parents. Artificial pollination may be a remedy for the low natural fruit set resulting in bunch failure. The extent of the problem associated with *E. oleifera* × *E. guineensis* hybrid is highly variable between progenies (Arnaud 1980; Schwendiman et al. 1982, 1983). In contrast, strikingly high natural fertility has been observed in interspecific hybrids of Suriname descent (Closen 1987; Rao et al. 1989).

Consequent upon the recognition of the importance of *E. oleifera* × *E. guineensis* interspecific hybrid, in vitro embryo rescue methods have been tested with success (Alves et al. 2011a, b).

11.2.11 Transfer of Alien Genes in Oil Palm via Backcross Breeding

Backcross breeding is an important method for the introgression of desirable genes from a relatively unimproved genotype into a recurrent host genotype. A series of backcross breeding programmes are in progress to introgress the desirable traits (oil quality, dwarfness and disease resistance) of *E. oleifera* into the best known combinations of *E. guineensis* genotypes (Obasola et al. 1977; Tam et al. 1977; Sterling et al. 1988; Sharma and Tan 1990; Le Guen et al. 1993; Chin 1993; Din and Rajanaidu 2000; Soh 1999; Sharma 2000). This method of alien gene transfer although takes a long time, has merits in terms of maintaining the advantageous genetic background of the recurrent parent.

In addition, backcross breeding method has been used in the development of AVROS and the Dumpy.AVROS in developing the AVROS and to introgress the dwarf trait in the Dumpy Deli to AVROS population, respectively. However, oil palm, being a perennial, backcrossing in the strict sense to the original recurrent genotype is not possible, rather it is done to the population or the progeny of the recurrent parent.

11.2.12 Prospects of Alien Gene Transfer in Oil Palm via Transformation

Gene introgression via genetic transformation is the recent and the most advanced method of alien gene transfer. It offers the ultimate potential for the introgression of genes from species that are not only distant but also quite far apart members in the plant kingdom. The perfected protocols of tissue culture methods have provided the basis for research into genetic transformation of oil palm. The first report on the transient expression in oil palm was by using the biolistics approach (Alves et al. 2011a). Studies on genetic transformation in oil palm have made significant progress since then. Te-chato et al. (2002), Zubaidah and Siti Nor (2003), Rohani et al. (2003), Adam et al. (2005) and Lee et al. (2006) reported work on establishing conditions for transformation. The genes needed to achieve genetic transformation for oil synthesis was reported by Shah and Cha (2000) and Asemota and Shah (2004), for carotenoids by Khemvong and Suvachittanont (2005) and for kernel expression by Cha and Shah (2001).

The transformation methods, direct gene transfer and *Agrobacterium*-mediated approaches have been used to transfer useful genes such as cowpea trypsin inhibitor (CpT1) (Abdullah et al. 2003) and *Bacillus thuringiensis* (*Bt*) crystal insecticidal protein genes as a solution for the problems caused by insect pests (Sharma 2000; Sharma et al. 2002) and also chitinase to address the problems related to basal stem rot. The synthetic gene *Cry1A(b)* has been successfully transformed using particle bombardment method and the expression tested (Lee et al. 2006).

Cry1A(b) synthetic gene is intended against the damages caused by Lepidopteran pests (Masson et al. 1999; Reardon et al. 2004). However, despite much advancement made in the genetic transformation in oil palm, this method is still not in practice for the production of planting material. Most Southeast Asian countries, where the oil palm is planted in massive scale, are still in the process of developing biosafety mechanisms for the planting of genetically modified crops. Therefore, it will take more time for the beneficial effects of alien gene transfer via genetic transformation to come into effect in oil palm.

11.3 Coconut

Beginning from the early twentieth century to about the 1960s, coconut oil occupied the topmost position as a vegetable oil in the tropics. Lately certain health concerns, raised due to coconut being saturated oil, reduced its value as a cooking oil in the international trade. While these concerns are currently been reinvestigated, the value of coconut in many of the coconut-growing countries remains undiminished as the term "tree of life" that had been tagged onto the coconut palm remains intact to date. Coconut palm is identified as a plant having a potential for alleviating poverty in areas where coconut is grown due to its multitude of uses in addition to being an oil crop. Coconut can be grown both in plantation scale as well as a home garden crop making it possible to be used in small scale or cottage industries for its diverse uses. Copra and oil, desiccated coconut, coir and fibre products, charcoal and other shell products are the important products from a coconut tree, while tender nut beverage coconuts, various uses of coconut leaves or fronds and timber industry are some other examples for the diverse uses of coconut.

11.3.1 Origin

Several conflicting theories regarding the origin and domestication of coconut have been put forward. A New World origin of coconut with subsequent dispersal to Asia and Polynesia has been proposed by several groups (Guppy 1906; Cook 1910; Ridley 1930; Bruman 1944). In these, the centre of origin of Cocoid palms, the closest relative of coconut, is North-Western and South America. Evidence from fossils and archaeological studies indicate a South West Pacific origin of coconut (Child 1974; Purseglove 1965), while Indian fossils and the Madagascar forest coconut support an Indian Ocean origin (Harries 1995). Even at present the issue of origin of coconut is still not fully resolved. However, coconut is found extensively distributed throughout the tropics including Central and South America, East and West Africa, South and Southeast Asia and the Pacific islands. From its putative centre of origin, coconut has been disseminated to both east and west by floating in the sea and also by human dissemination (Ohler 1984).

11.3.2 Biology/Botany

Coconut (*Cocos nucifera* L.) is a monocot belonging to the family Arecaceae and to the subfamily Cocoidae. Subfamily Cocoidae includes 27 genera and 600 species, and coconut is currently the sole species of the genus *Cocos*. Coconut possesses a diploid genome with 16 pairs ($2n = 2x = 32$) of chromosomes. Similar to oil palm, coconut is a monoecious plant bearing both male and female flowers on the same plant. However, unlike oil palm, coconut bears both male and female flowers on the same inflorescence which are produced in a continuous succession on the adult palms. A coconut palm upon reaching the reproductive stage produces about 12–15 inflorescences per year throughout its economic lifespan. Coconut inflorescence consists of many flower-bearing spikelets situated on a central axis or a peduncle. The female flowers are located at the base of the inflorescence while a numerous number of male flowers occur along the length of the spikelets. Tall varieties of coconut are predominantly outbreeding due to nonoverlapping of the male and female phases of the inflorescence. The dwarf or short varieties of coconut are naturally self-pollinating due to the overlapping of the two phases. The important phenomena of the inflorescences with respect to the genetic improvement of the palm are the lack of incompatibilities and the ability to artificial cross-pollination as well as the artificial self-pollination.

11.3.3 Varietal Groups/Populations of Coconut

A variety is defined as "a general term to denote a single strain or a group of strains which distinctly differ in structural or functional characters from one another or a group of the same species which can be depended upon the ability to reproduce itself true-to-type" (Menon and Pandalai 1958). Many reported types of coconuts in the world may not qualify as distinct varieties according to the discrete considerations in the definition. However, there are many different varieties, forms and types of coconut recorded in the world, although the terminology may not be exact or strictly correct in the different coconut-growing countries or regions within a country.

Although different classifications of coconut exist, there is one common feature in all of them, the grouping of coconut as talls and dwarfs. Talls have their specific characteristics and have gained popularity as commercial planting materials among the unimproved planting material of coconut throughout the world. Dwarf was later identified as a variety with a high potential to be used as a parent to transfer favourable genes to commercially grown tall coconuts. In certain countries a few different intermediate coconut varieties, which display characteristics in between the talls and the dwarfs, have also been reported. King coconut in Sri Lanka (Liyanage 1958), Niu Leka Dwarf in Fiji (Bourdeix et al. 2005a) and Gangabondom in India (Menon and Pandalai 1958) are few of the examples for this semi-tall/semi-dwarf or intermediate groups of coconut. A comparison of contrasting features of the different types of coconuts is presented in Table 11.2.

Table 11.2 Comparison of different varieties of coconuts

Trait	Tall	Dwarf	Intermediate
Palm height	Tall (20–30 m)	Short (10–15 m)	Vary from short to tall
Trunk circumference	Enlarged with a bulbous base	Thin and no bole formation	Usually enlarged with a root bole
Lifespan	60–100 years	40–50 years	40–60 years
Time taken for flower initiation	5–8 years	2.5–4 years	Vary from 4 to 6 years
Mode of pollination	Highly crossed	Highly selfed	Highly selfed
Bearing nature	Continuous	Seasonal	Seasonal to continuous
Nuts/palm/year	Average 40–60	Average 80–100	Average 50–100
Whole fruit size	Very small to large	Very small to medium	Medium to large
Copra amount and quality	200 g/nut; good	80–100 g/nut; inferior	100–150; Mainly inferior
Leaf and bunch attachment	Strong	Fragile	Usually fragile
Pigmentation of nuts	Mixture of green, brown and yellow	Pure green, brown, yellow or red	Green, red

The main groups of coconut include palms of different morphologies despite their basic similarity in stature and the pollination behaviour. For example, the talls include morphotypes which show differences in nut colour and size, shell thickness, mesocarp or endocarp differences. Liyanage (1958) referred to these as forms within varieties and Bourdeix et al. (2005a,b) referred to them as phenotypically distinct variants.

11.3.4 Goals in Coconut Breeding

Similar to other crops high yield is the primary objective of the genetic improvement of coconut. Coconut has diverse uses but the main economically important yield parameter is the weight of copra or kernel. Fruit and nut components, number of nuts and the weight of kernel per nut are the determining factors of per palm coconut yield. However, there is a negative correlation between the number of nuts and the size of the nuts in a bunch. Consequently, improvements towards increasing the rate of inflorescence (resulting in bunch) emission will be one way of overcoming this problem. Efforts to maintain a balance between nut number and the nut size will be another approach to increase the kernel/copra yield.

Precocity or the shortening of the vegetative phase is one major objective in coconut breeding. Coconut is a perennial plant with a long vegetative phase as well. As a result of the long vegetative phase, which lasts for about 6–7 years, the growers' waiting period for returns to their investments also becomes long reducing the appeal of coconut as an economic venture. The genetic potential of the dwarf coconuts for early flowering and bearing provides the required genetic material for breeding for precocity.

With the global scenario of climate change and the occurrence of prolonged droughts, the attention of the coconut breeders has shifted on breeding coconuts for tolerance to abiotic stresses, water stress being the most important. Coconut is a monocot with a fibrous root system which does not grow into deeper layers in the soil resulting in adverse effects from moisture stress than the dicot perennials having a tap root. Hence, many countries are now focusing on breeding for tolerance to moisture stress in their coconut breeding programmes to expand the cultivation of coconut to newer areas and also for higher productivity of the existing plantations.

Breeding for tolerance to biotic stresses (pests and diseases) is a highly beneficial way of managing and controlling them. So far the most devastating groups of pathogens of coconut are the phytoplasmas and viroids which are intracellular, causing incurable deadly or debilitating diseases. The phytoplasma group is recorded to be the causal organism for diseases such as lethal yellowing in Africa, Kalimantan wilt in Indonesia and Kerala Root wilt in India, while Cadang-cadang in Malaysia is reported to be caused by viroids. The countries plagued by phytoplasma diseases include tolerance breeding for such diseases as a primary objective in their coconut breeding programmes (Nair et al. 2006).

Development of short stature of the palms is another objective of coconut breeding. Most of the old coconut stands are very tall and the scarcity of labour for picking has become a major problem at present even in large plantations in many of the coconut-growing countries. Furthermore, short stature is an important character in cultivars for home gardens also.

11.3.5 Genetics and Breeding of Coconut

Coconut is a difficult to breed perennial plant. Long generation interval, cross-pollination breeding behaviour of tall coconuts resulting in highly heterogeneous populations, lack of a viable vegetative propagation method, low number of seeds produced per palm and the massive stature of the palm are the most important constraints in coconut breeding. Despite these limitations coconut breeding programmes have been started from the first quarter of the twentieth century. In these programmes two breeding methods, mass selection and hybridization, have been widely used for the genetic improvement of the coconut palm.

The majority of the coconut plantations in the world are derived from mass selection. Mass selection was scientifically recognized as the most fundamental method for coconut breeding (Liyanage 1955). The common selection criteria was the yield of copra per palm or one of its components such as number of fruits produced or per nut copra content. In many of selection programmes, seednuts were selected mainly based on the size, weight and shape of the nuts. The resulting coconut populations ware subjected to successive selection for fruit characteristics. Around the mid-twentieth century, the system of selection of the best trees within the best plots was applied, and coconut breeders throughout the world adopted this system of mass selection. Three methods of mass selection have been practiced depending on

the reproduction system used: mass selection using open pollination, selfing or intercrossing between parents.

Intervarietal hybridization has been widely practiced in coconut to extract heterosis from genetically diverse material. Much progress has been achieved with this method world over mainly with respect to nut yields, precocity and copra productivity.

11.3.6 Alien Gene Transfer in Coconut

Coconut is the sole species of the genus Cocos. Because of this the alien gene transfer at interspecies level is not possible in coconut. However, hybridization between varieties and different populations has been extensively practiced with success to transmit the characters of interest to cultivated material.

The main coconut varieties tall and dwarf are the genetic material much used in hybridization to mix important traits. Out of these two varieties, tall has been the economically important commercial planting material. The tall coconuts are hardy and tolerate the harsh environments better than the dwarf varieties. In contrast the dwarfs display a certain amount of seasonality in bunch emission and nut setting upon pollination. The talls are usually regular and continuous bunch bearers, although a reduction in nut number is observed during prolonged drought periods. The nuts of the talls also are bigger and produce more copra per nut (Table 11.2). In comparison, dwarfs produce a larger number of nuts per year. However, one of the most troublesome inferiorities in the commercially preferred tall coconut varieties is the long vegetative phase which lasts for about 5–8 years. In comparison, the dwarfs are precocious coming into bearing in about 2–4 years. Another important advantage of dwarf coconuts is their short stature compared to tall coconuts. Coconut hybridization programmes world over have focused on transmitting the economically important traits precocity, high nut number and shorter stature from the dwarf varieties to the commercial tall cultivars through hybridization between the tall and dwarf varieties.

Tall coconuts in the world are reported to be of two types. The first is the populations of talls in the Southeast Asian and the Pacific origin. The nuts of these tall coconuts are generally larger and round in shape. The second type is the tall coconuts present in South Asia and the African region. The nuts of these tall coconut populations are generally smaller and elongated in nut shape than the Pacific talls. On the other hand the dwarf coconuts are believed to have evolved from the tall coconuts of the Southeast Asian and the Pacific region (Perera et al. 2000). Even within the two tall coconut types and the dwarf coconut type, there are many different populations and/or subpopulations which are mainly geographically isolated and distinguished. As such the accepted international naming most of the times includes the country or the region where a particular coconut type, variety or a population, has been grown. Sri Lanka Tall, San Ramon Tall, West African Tall and Fiji Tall are some examples for such naming in tall coconuts, while Malayan yellow

Dwarf, Sri Lanka Yellow Dwarf and Madang Brown Dwarf are some examples for dwarf coconuts. These different populations show morphological and genetic variations, and they do provide the necessary raw material for transfer of important genes in coconut.

11.3.7 Achievements and Impacts of Alien Gene Transfer in Coconut

Different types of intervarietal hybridizations have been carried out to transfer the economically important traits to commercially cultivated varieties of coconut. The most common intervarietal coconut hybridization has been between the tall and the dwarf varieties (D×T crosses). Hybridization between the tall and the dwarf is considered a wide cross in coconut because wider crosses, such as interspecies hybridizations, are not feasible due to coconut being the sole species of the genus Cocos. Considerable achievements with respect to the goals of coconut breeding have been made even with the intervarietal hybridizations between the talls and the dwarfs in many coconut-growing countries.

The coconut hybrids Mawa (PB 121—cross between Malayan Yellow Dwarf and West African Tall), Camren (PB 113—cross between Cameroon Red Dwarf and Rennell Island Tall) produced in Ivory Coast, Matag (PCA 15-2 cross between Malayan Red Dwarf and Tagnanan Tall) produced in the Philippines, CRIC 65 (cross between Sri Lanka Green Dwarf and Sri Lanka Tall) produced in Sri Lanka and Maren produced in the Solomon Islands are a few examples of widely planted tall and dwarf hybrids. In all these intervarietal hybrids, the characters, viz., high nut number, precocity and shorter stature, have been transmitted to a certain extent to the tall coconut varieties (see Fig. 11.2). These improved cultivars have been mass produced and widely cultivated recording yield increments varying from about 50 to 150 % than the tall cultivars. In addition, the tall and dwarf hybridization has also resulted in shortening the vegetative phase by about 30–50 % depending on the parents used. Also these hybrids are shorter in height compared to their tall parents increasing their appeal to growers (Bourdeix et al. 2005a, b).

Tall×tall hybridizations between populations of different origins have also been carried out in coconut in several countries. The coconut hybrid Waren or PB 213 is produced by crossing the two different tall varieties, West African Tall and Rennell Island Tall. This cultivar is tall in habit and flowers earlier than its two tall parents in addition to producing larger nuts. The improved cultivar Wavan or PB 214 is yet another tall×tall hybrid produced by crossing two different tall populations West African Tall and Vanuatu Tall. This hybrid has also brought precocity to a certain degree to tall cultivars in addition to its comparable yields. CRISL 98 is yet another tall×tall hybrid produced in Sri Lanka. The parents, Sri Lanka tall and San Ramon tall, are two populations of tall from South Asian and Southeast Asian origins and are therefore considered a wider cross than two tall populations of the same group.

Fig. 11.2 A dwarf × tall coconut hybrid in Sri Lanka

With transmittance of genes for higher copra content and high nut weights from San Ramon, by now CRISL 98 is the highest per nut copra producer in Sri Lanka (Perera et al. 2010).

11.4 Conclusion

As has been discussed above, remarkable achievements have been made in increasing the quantity and quality of palm oil, tolerance to biotic stresses and reducing the stature of oil palm cultivars through gene transfer between populations of different origins and gene transfer through wider crosses and interspecies hybridizations in oil palm. Similarly, in coconut also much advancement has been made with respect to yield components, precocity and shorter stature using intervarietal and interpopulation gene transfer methods. These gene transfer methods have helped to bring oil palm to the topmost position in the world oil trade and coconut to provide vegetable oil and livelihoods to millions of people in the coconut-growing countries. Nonetheless, keeping in view that avenues for interspecific hybridizations and consequently alien gene transfers are very limited, particularly in coconut, efforts need to be initiated towards development of transgenics. Molecular tools also need to be integrated with conventional breeding in these two most important oil crops in order to hasten the breeding process as well as develop cultivars with specific traits. In general, as compared to other commercial crops, genomic resources are a limitation in oil palm and coconut, and this issue needs to be addressed on priority to initiate a target-oriented and ameliorated breeding for improvement of oil palm and coconut.

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Chapter 12
Groundnut

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Abstract Groundnut or peanut is an important legume nut known for its multifarious uses including oil production, direct human consumption as food and also animal consumption in the form of hay, silage and cake. Being a grain legume, peanut has an important nutritional value for human beings, and its nutritional value has been exploited for combating malnutrition in children. The breeding objectives in groundnut focus on increasing yield, incorporating resistance/tolerance to biotic and abiotic stresses and improving oil and nutritional quality including safety of its consumption by humans and animals. However, limited genetic variability in the cultivated germplasm and difficulties in hybridisation have slowed down the progress in groundnut breeding. The wild relatives are considered as sources of several agriculturally important traits including resistance to pests and pathogens, tolerance to abiotic stresses and variable nutritional value. These resources have been used in groundnut breeding programmes for improving the above traits, simultaneously addressing the constraint of reproductive barrier in successful hybridisation arising due to different ploidy levels of *A. hypogaea* and its wild relatives. This has been achieved through different routes: the hexaploid pathway, two different diploid/tetraploid pathways and genetic engineering-based methods. Nonetheless, the use of wild introgressions in groundnut improvement programmes has not been up to the desired extent, and therefore concerted efforts for a large-scale generalised introgression programme are required. This chapter discusses the evaluation and utilisation of alien introgressions in groundnut improvement, the achievements made hitherto and the future strategies for initiating a large-scale introgression programme.

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12.1 Introduction

Groundnut, also commonly known as peanut (*Arachis hypogaea*), is a tropical legume mainly grown to produce oil and for human and animal consumption. Peanut is grown in about 120 countries in the world in a total area of 24.6 million ha, with a world production of 38.2 million tonnes (Mt). Asia is the major peanut-producing region in the world. In this region, China and India are the major contributors with 15.7 and 5.6 Mt in 2010, respectively (FAOSTAT 2010). Africa ranks second in the world peanut production. In this region, Nigeria (2.6 Mt), Senegal (1.2 Mt) and Sudan (0.7 Mt) are the major producing countries (FAOSTAT 2010). In Africa and Asia peanut is mainly grown by resource-poor farmers. In the Americas, the USA and Argentina are the major producing countries with 1.8 and 0.6 Mt in 2010, respectively.

Peanut yields vary drastically between regions and between countries within a region. Although Africa is the second region in terms of production, it has the lowest yield (1 t/ha on average) as compared to Asia (1.8 t/ha) and to the Americas (3 t/ha). In West Africa, peanut yields vary from 0.5 t/ha in Niger to 1 t/ha in Senegal and can reach up to 1.5 t/ha in Nigeria. In Asia yields vary from 1.5 t/ha in India to 3 t/ha in China. The low peanut yields observed in many countries in Africa and Asia are related to rainfed and low-input growing conditions. In these countries where the rainfall pattern is irregular, peanut is often subjected to drought.

Worldwide peanut production is principally dedicated to oil and food products. Between 1996 and 2000, 49 % of world production has been used for oil and 41 % as food product components (Revoredo and Fletcher 2002). Peanut is also used for feed through the valorisation of oil cakes that represent an interesting source of proteins for livestock. In most Sahelian countries, groundnut straw is also used as dried hay and represents a major source for cattle feed during the dry season. As is the case with most of the grain legumes, peanut has an important nutritional value for human consumption. Several studies have reported a positive impact of peanut on human health, and its nutritional value has been exploited for the elaboration of highly nutritious food products used in the treatment of severe child malnutrition (Briend 2001).

Peanut breeding objectives are mainly focused at increasing yield and improving resistance to foliar diseases and nematodes, tolerance to drought, quality of oil and food and safety (resistance to aflatoxin contamination and reduced allergenicity). Significant progress has been achieved in developing elite cultivars using sources of adaptive traits and disease resistance that exists in cultivated germplasm collections. This was particularly the case for drought tolerance-related traits, oil quality and resistance to rosette disease. However, for some other traits such as resistance to early and late leaf spot, rust and nematode, only moderate levels of resistance are observed in the cultivated germplasm (Holbrook and Stalker 2003).

Peanut breeding has also been slowed down by the difficulties in making large numbers of crosses and by the low number of progenies produced per cross. This has limited the exploration and the utilisation of cultivated genetic resources. In addition to these practical constraints, and in spite of the morphological variability that is observed in the cultivated gene pool, there are limitations to genetic improvement that can be achieved using only cultivated germplasm. A clear example of this is disease resistance: wild species display much stronger disease resistances than are found in cultivated peanut. There are also good theoretical reasons to believe that genetic limits for more complex traits like yield and drought tolerance can be overcome by using wild relatives of the crop as this has been the case in other crops (Gur and Zamir 2004). For these reasons, peanut breeders have for many years been interested in the introduction of new alleles from wild species.

12.2 Peanut Gene Pools and Genetic Resources

The genus *Arachis* consists of 80 described species (Krapovickas and Gregory 1994, 2007; Valls and Simpson 2005) and is divided into nine taxonomic sections: *Trierectoides*, *Erectoides*, *Procumbentes*, *Rhizomatosae*, *Heteranthae*, *Caulorrhizae*, *Extranervosae*, *Triseminatae* and *Arachis* (Fig. 12.1). These divisions were made

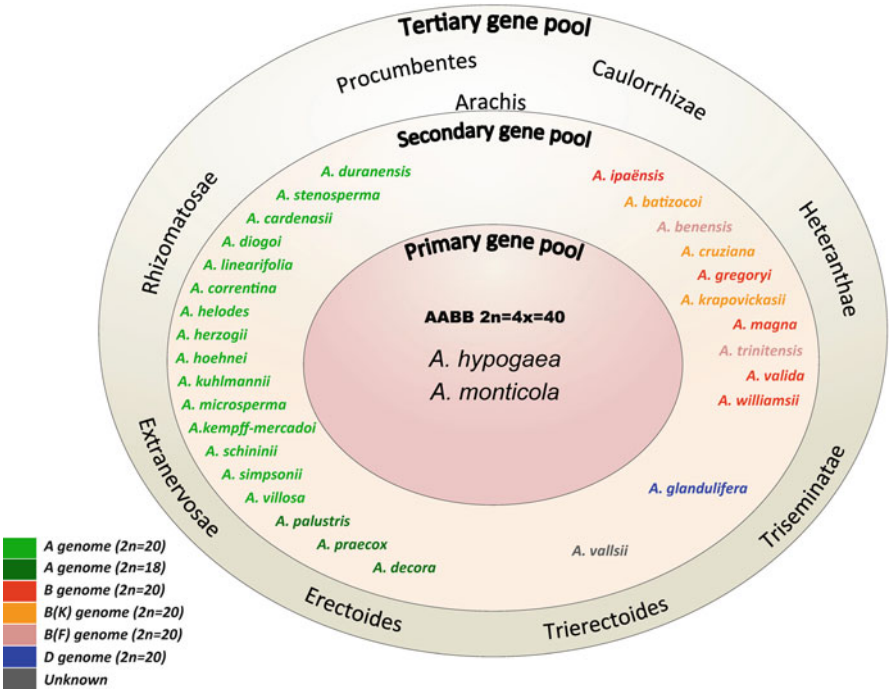

Fig. 12.1 Primary, secondary and tertiary gene pools of the genus *Arachis*

based on sexual compatibilities, morphological and cytogenetic features and geographic distributions (Krapovickas and Gregory 1994). The sexual compatibility data available from a large number of crossing experiments is very informative as to the barriers between gene pools in the genus (Krapovickas and Gregory 1994). The section *Arachis* contains the primary gene pool of cultivated peanut with two tetraploids, *A. hypogaea* and *A. monticola*. ($2n=4x=40$; genome AB) and the secondary gene pool with the most closely related wild species.

Arachis hypogaea presents considerable morphological variation, and two subspecies, *hypogaea* and *fastigiata*, have been described (Krapovickas and Gregory 1994). Subspecies *hypogaea* has spreading growth habit with side branches procumbent to decumbent, a long growth cycle, no flowers on the central stem and regularly alternating vegetative and reproductive side stems. This subspecies is divided into two botanical varieties *hypogaea* and *hirsuta*, the latter being distinguished by more hirsute leaflets and even longer cycle. These varieties, respectively, exemplify "Runner" and "Peruvian Runner" agronomic classes. The subspecies *fastigiata* Waldron has a more erect growth habit with side branches erect to procumbent, a shorter cycle, flowers on the central stem and reproductive and vegetative stems distributed in a disorganised fashion. This subspecies is divided into four botanical varieties, *fastigiata*, *vulgaris*, *aequatoriana* and *peruviana*. The former two are by far the most economically important and exemplify the agronomic classes "Spanish" and "Valencia", respectively (Krapovickas and Gregory 1994).

Within the agronomic classes, modern cultivars are relatively uniform compared to landraces. Especially in South America, but also in Africa and Asia, landraces are spectacularly diverse. This diversity provides a source for constant study, such as the recent interesting description of 62 distinct landraces in Bolivia (Krapovickas et al. 2009). Also, new collections of landraces continue to be made. For instance, in the Xingu Indigenous Park in the Central-West of Brazil, the Kayabi people cultivate peanuts which are morphologically very diverse, displaying combinations of unusual characters which make them unique. Some types form very large plants and have a very long cycle; some have extremely large seeds. The different types also display diverse seed colours and patterns, purple, brown, red or white, variegated or uniform in colour (Freitas et al. 2007; Bertioli et al. 2011).

Morphological diversity is so high that a different origin for the two subspecies was proposed. This hypothesis was supported by the partial reproductive isolation of the two subspecies (Singh and Moss 1982; Lu and Pickersgill 1993). However, molecular data has firmly contradicted this hypothesis. Genetic variability observed among commercial cultivars and landraces of peanut is so low that it is generally accepted that peanut is an allotetraploid of recent and single origin (Halward et al. 1993; Kochert et al. 1996; Raina et al. 2001; Milla et al. 2005).

The secondary gene pool includes *A. hypogaea*'s most closely related wild species that can be used for peanut crop improvement. Most of these species are diploid ($2n=2x=20$) with metacentric chromosomes of similar size (genomes A, B, F and K); one species (*A. glandulifera*) is diploid with an asymmetric karyotype (genome D); three can be considered dysploid ($2n=2x=18$) (Krapovickas and Gregory

1994; Lavia 1998; Peñaloza and Valls 1997; Stalker 1991; Valls and Simpson 2005; Robledo and Seijo 2010). The single wild tetraploid species, *A. monticola*, is very closely related to *A. hypogaea* (Lu and Pickersgill 1993), probably sharing the same origin, and it is considered *A. hypogaea*'s immediate tetraploid ancestor (Seijo et al. 2007; Grabiele et al. 2012). The ploidy barrier between cultivated peanut and most of its wild relatives (with the single exception of *A. monticola* $2n=40$) effectively prevented the introgression of wild genes into cultivated peanut and created a strong genetic bottleneck.

The most frequent of the genome types among the species is the A genome (Fig. 12.1). It is characterised by the presence of a chromosome pair of reduced size and by chromosomes with strongly condensed centromeric bands (Husted 1936; Seijo et al. 2004). The next most frequent genome type is B, which is characterised by the lack of a small chromosome pair and by chromosomes with a much lower degree of centromeric DNA condensation. The genome types F and K were formerly considered B genome species, and their recent classification was based on rDNA loci and the presence in most chromosomes of strongly condensed centromeric bands (Robledo and Seijo 2010). Phylogenies based on DNA sequence data strongly support the validity of these genome divisions (Moretzsohn et al. 2004, 2013; Milla et al. 2005; Tallury et al. 2005; Bravo et al. 2006; Bechara et al. 2010; Friend et al. 2010; Grabiele et al. 2012).

12.3 Characterisation of Wild Species

Peanut wild relatives are considered as sources of agriculturally important traits that can be tapped to improve the cultigen. Although a low number of peanut wild accessions have been used in the breeding programmes, as compared to the high diversity that exists in wild species, extensive phenotypic characterisation of peanut wild relatives has been performed for many traits.

12.3.1 Resistance to Pests and Pathogens

Resistance to pests and pathogens have been identified in several species belonging to the genus *Arachis* (Upadhyaya et al. 2011b). When crosses with the cultivated species are possible, wild species can be used as a source of disease resistance for cultivated peanut, thanks to their broad resistance spectrum and because they are effective against a disease for which variability is not available in the cultivated species. One good example of a source of resistance that fits these criteria is the accession GKP10017 (PI 262141) of the wild species *A. cardenasii* that has been used in the US breeding programmes to transfer resistances for both early and late leaf spot, nematodes and insects into cultivated species. Moreover, Singh and Oswalt (1991) reported accessions of *A. duranensis*, *A. stenosperma*, *A. cardenasii*

and *A. villosa* combining immunity and/or resistance to rust and early or late leaf spot, tomato spotted virus, thrips and/or aphids.

Resistance to disease might have complex inheritance that involves several components including initial infection, lesion size and number, sporulation and defoliation. Recently, Mallikarjuna et al. (2012a) characterised several diploid species, their hybrids and auto- and allotetraploid derivatives. They reported that they combined several components of resistance to late leaf spot. Leal-Bertioli et al. (2009) phenotyped an F_2 population derived from the cross between two wild diploid species *A. duranensis* and *A. stenosperma* for resistance to late leaf spot and expressed the results as percentages of diseased leaf area (DLA). The susceptible parent had an average of 4.53 % DLA, and the resistant parent had 0.15 % DLA. Among the 93 F_2 plants, 73 had lower percentage DLA than the resistant parent, of which 47 had no lesion attesting for important transgressive segregation. Finally, five QTLs of resistance to late leaf spot were detected. Moreover, resistance for foliar diseases was characterised at the microscopic level, and it was found that in *A. stenosperma*, resistance occurs at the pre-penetration level (Leal-Bertioli et al. 2010). The same species also harbours resistance against root-knot nematode, a resistance that is manifest in at least two distinct levels: very few nematodes that penetrate the roots and those that are unable to set up an infection because of a hypersensitive-like response (Proite et al. 2008).

Despite the high level of disease resistance generally found in peanut wild relatives, important variation can exist between accessions belonging to the same species. Singh et al. (1996) conducted a detailed characterisation of 42 accessions belonging to the species *A. duranensis* using principal component analysis and showed that differences in reaction to rust between accessions of the same species explained 15 % of the total variation. Variation between accessions for resistance to pests and diseases has also been reported for nematodes (Sharma et al. 1999), peanut bud necrosis virus (Reddy et al. 2000), late leaf spot and rust (Pande and Rao 2001). These results indicated that clear identification of most resistant wild accessions is needed before their utilisation as source of disease resistance.

12.3.2 Tolerance to Abiotic Stresses

Unlike resistance to pests and diseases that is generally governed by one or a few genes, tolerance to abiotic stress is often polygenic, subject to G×E interactions and thus necessitates accurate phenotyping to better capture the genetic component of trait variation. Field evaluation of peanut wild relatives over several years is difficult because of their differences in generation time (annual to perennial life cycles) and the difficulty of harvesting seeds. Hence, only a limited number of studies have reported the characterisation of peanut wild relatives for response to abiotic stress, and most of them were based on the measurement of morphological and/or physiological traits in greenhouse conditions. Nautiyal et al. (2008) evaluated 38 accessions of 12 *Arachis* species belonging to four sections for thermo-tolerance (heat and cold).

Several morphological and physiological traits, viz., leaf morphology, electrolyte leakage, leaf water potential, specific leaf area (SLA) and leaf chemical constituents, were recorded. Tolerance to heat and cold has been expressed as percentage of leaf relative injury (RI). These authors reported important variation between species and between accessions within a species. One accession of *A. glabrata* (section *Rhizomatosae*) and one accession of *A. paraguariensis* (section *Erectoides*) were identified as heat resistant and cold resistant, respectively. One accession of *A. appressipila* (section *Procumbentes*) was found susceptible to both cold and heat. Moreover, positive correlations were found between RI and SLA both for heat and cold resistance, indicating that genotypes with thicker leaves also have higher tolerance. Upadhyaya et al. (2011a) measured 41 morpho-agronomic traits over 269 accessions from 20 wild *Arachis* species belonging to six sections. A large range of variation was observed for traits related to drought: earliness, SLA and SPAD chlorophyll meter reading (SCMR). Finally, a set of 20 accessions with superior agronomic and drought-related trait combinations were proposed to be used in breeding programmes for introgression of wild favourable alleles in the genetic background of cultivated varieties. Leal-Bertioli et al. (2012) investigated drought-related traits such as leaf morphology, transpiration profile, SCMR, SLA and transpiration rate per leaf area of two wild diploid species (*A. duranensis* and *A. ipaënsis*) and one synthetic tetraploid deriving from the cross between the same diploid species. One interesting result that came from this study is that most drought-related traits such as leaf area, stomata size and transpiration rate were substantially modified when evaluated in a wild tetraploid context attesting for effects of the ploidy level on trait variations. These authors concluded that, for the introgression of drought-related traits from wild species to cultivated species, evaluation of the synthetic tetraploid was likely to be more informative than the evaluation of the diploids. Additionally, it would also be interesting to investigate the effect of genetic background (cultivated versus wild) on the modification of drought-related trait variation.

12.3.3 Evaluation for Nutritional Value

As peanut is an important food and oil crop, improving protein content and oil quality in seeds are important objectives of breeding programmes. The properties of peanut oil are determined by the fatty acid content and particularly the oleic-to-linoleic ratio (O/L). The analysis of the chemical composition of peanut wild species seeds has been reported. The oil and protein content and the fatty acid and sterol composition of the seeds of several wild accessions were studied by Stalker et al. (1989) in sections *Arachis*, *Heteranthae*, *Caulorrhizae* and *Procumbentes* and by Grosso et al. (2000) in sections *Arachis*, *Extranervosae*, *Erectoides* and *Triseminatae*. The highest oil content and O/L ratio were found in species belonging to section *Arachis* (*A. stenosperma* and *A. villosa*, respectively). In both studies, none of the wild species analysed overrode the cultivated species in terms of chemical quality and oil stability. However one can expect that the cultivated species could benefit from

positive alleles from the wild through transgressive segregation. Jiang et al. (2009) evaluated 87 wild *Arachis* accessions and 113 interspecific offspring for traits related to fatty acid composition. These authors reported transgressive segregation in progeny derived from the crosses between *A. stenosperma* and two Chinese cultivated varieties and between *A. glabrata* and one cultivated line. Two progenies involving *A. stenosperma* as wild donor had 12.8 to 29.7 % more oleic acid than their parents. Similarly, four progenies from the cross between *A. glabrata* and one cultivated line had higher content of oleic acid and lower content of palmitic acid than the cultivated line. Moreover, in this study, high content of oleic acid was found in accessions of *A. duranensis* and *A. pusilla*. Wang et al. (2010) evaluated 39 wild species of different sections for oil content, fatty acid composition and D150N functional mutation of the *FAD2A* gene. Significant variability was found among species, but no accession had high oleic/linoleic acid ratio. Finally, Upadhyaya et al. (2011a) evaluated the nutritional value (oil, protein and sugar) of 20 peanut wild accessions among which seven belong to *A. stenosperma*, three each to *A. monticola* and *A. pusilla*, two to *A. kuhlmannii* and one each to *A. villosa*, *A. batizocoi*, *A. duranensis*, *A. dardani* and *A. paraguariensis*. These authors reported for oil content a range of variation (45–55 %) similar to that reported in cultivated varieties.

12.4 Molecular Markers and Maps: The Introgression Toolbox

Plant breeding programmes generally use backcrossing for the introgression of wild genes into elite materials for a specific trait. At each backcross generation, plants with the wild target phenotype introgression are selected, while the background of the cultivated parent is recovered through generations. Without the help of molecular markers, this process results in the introgression of a large portion of the donor genome, which carries undesirable genes associated to the allele of interest (linkage drag), and many backcross generations are necessary to eliminate the deleterious genes. Molecular markers, ordered on a genetic map, provide a tool to monitor the size and distribution of wild introgressions throughout the breeding process. Their availability is a key step in the successful implementation of large-scale introgression programmes.

12.4.1 Molecular Markers

The first markers used in peanut were isozymes and proteins (Grieshammer and Wynne 1990; Krishna and Mitra 1988; Lu and Pickersgill 1993), followed by restriction fragment length polymorphisms—RFLPs (Kochert et al. 1991, 1996; Paik-Ro et al. 1992), random amplified polymorphic DNA—RAPD (Dwivedi et al. 2001; Halward et al. 1991, 1992; Hilu and Stalker 1995; Raina et al. 2001;

Subramanian et al. 2000) and amplified fragment length polymorphism—AFLP (Gimenes et al. 2002; He and Prakash 1997, 2001; Herselman 2003; Milla et al. 2005; Tallury et al. 2005). However, none of these marker systems were very informative in cultivated germplasm. In recent years, microsatellite or simple sequence repeat (SSR) markers have become the assay of choice for genetic studies in *Arachis*, since they are multiallelic, co-dominant, polymorphic, transferable among related species, PCR based and usable in tetraploid genomes. In consequence, efforts have been made by several research groups to develop microsatellite markers for peanut. Up to 15,000 SSR markers have been published to date (Hopkins et al. 1999; Palmieri et al. 2002, 2005; He et al. 2003, 2005; Ferguson et al. 2004; Moretzsohn et al. 2004, 2005, 2009; Bravo et al. 2006; Budiman et al. 2006; Gimenes et al. 2007; Proite et al. 2007; Wang et al. 2007, 2012; Cuc et al. 2008; Gautami et al. 2009; Guo et al. 2009; Liang et al. 2009; Nagy et al. 2010; Song et al. 2010; Yuan et al. 2010; Koilkonda et al. 2012; Macedo et al. 2012; Shirasawa et al. 2012a). The availability of a great number of microsatellite markers has enabled access to the low genetic variation available in cultivated peanut (Barkley et al. 2007; Krishna et al. 2004; Macedo et al. 2012; Tang et al. 2007; Varshney et al. 2009b). Recently, Shirasawa et al. (2012b) developed 535 markers derived from transposon-enriched genomic libraries. These MITE markers showed great potential, as they detected higher polymorphism levels than genomic microsatellite markers. Finally, single-nucleotide polymorphism (SNP) markers constitute the most abundant molecular markers in the genome and can be carried out with higher throughput genotyping methods. SNP markers have been widely used in many plant species. However, they have not been used in peanut so far, as the implementation in polyploid plants is difficult.

12.4.2 Genetic Maps

Linkage maps are particularly useful for the study of the genome structure and organisation and for marker-assisted selection in breeding programmes. Due to the very low genetic variation in cultivated peanut, interspecific populations have first been used for the construction of linkage maps in *Arachis*.

The first published map for *Arachis* was based on RFLP markers and developed using an F_2 population of 87 individuals derived from a cross *A. stenosperma* × *A. cardenasii*, both diploid species with A genome (Halward et al. 1993). One hundred and seventeen loci were mapped into 11 linkage groups covering a total map distance of 1,063 cM. A diploid backcross population derived from the same parents was also used to compute a linkage map (Garcia et al. 2005). One hundred and sixty-seven RAPD and 39 RFLP loci were mapped into 11 linkage groups, spanning 800 cM. The 39 RFLP markers were common to the F_2-based map of Halward et al. (1993) and were used to establish correspondences between both maps. All common markers mapped to the same linkage groups and mostly in the same order.

The first genetic map for the tetraploid genome of *Arachis* was based on a backcross population (BC_1) having the amphidiploid TxAG-6 [*A. batizocoi* ×

(*A. cardenasii* × *A. diogoi*)]^{4x} as donor parent and *A. hypogaea* cv. Florunner as recurrent parent (Burow et al. 2001). A total of 370 RFLP markers were mapped into 23 linkage groups covering 2,210 cM. The pairing of homoeologous linkage groups was consistent with the disomic nature of the allotetraploid peanut.

The first peanut SSR-based map was constructed for an F_2 population derived from a cross of two diploid species with A genome, *A. duranensis* and *A. stenosperma* (Moretzsohn et al. 2005). One hundred and seventy loci were mapped into 11 linkage groups covering 1,231 cM of total map distance. New markers were added to this map, resulting in 369 loci, including 188 microsatellites, 80 anchors and 35 resistance gene analogue (RGA) markers, mapped into ten linkage groups, as expected for diploid species of *Arachis* (Leal-Bertioli et al. 2009). The same authors developed a diploid F_2 population derived from the cross *A. ipaënsis* × *A. magna* to build a map for the B genome of *Arachis* (Moretzsohn et al. 2009). A total of 149 co-dominant markers, mostly microsatellites, were mapped into ten linkage groups spanning a distance of 1,294.4 cM. Fifty-one common markers evidenced the high synteny of the B genome map and the A map and revealed the translocation of chromosomal segments from the group A7 to the group A8.

A synthetic amphidiploid (*A. ipaënsis* × *A. duranensis*) was crossed and backcrossed to *A. hypogaea* cv. Fleur 11, resulting in 88 BC_1F_1 individuals (Fonceka et al. 2009). This population was used to develop an SSR-based linkage map for the tetraploid genome, composed of 298 markers and 21 linkage groups, covering a total distance of 1,843.7 cM. The segregation analysis indicated a disomic inheritance of all loci and chromosome pairing occurring between homologous genome confirming the close relationship between the wild diploids *A. duranensis*, *A. ipaënsis* and the cultivated peanut and highlighted structural rearrangements, such as chromosomal segment inversions and a major translocation event, between the A and B genome species. A comparative analysis of this map with the diploid genome maps of Moretzsohn et al. (2005, 2009) suggested the occurrence of this event prior to the peanut's tetraploidisation.

An intraspecific map was published in 2008, using 142 individuals of a recombinant inbred line (RIL) population derived from a cross between one accession of *A. hypogaea* subsp. *hypogaea* and one accession of the *fastigiata* subspecies (Hong et al. 2008). New markers were added to this map, and two additional maps were constructed based on RIL populations having accessions of the subspecies *fastigiata* as parents (Hong et al. 2010). Of the 901 screened, 132, 109 and 46 SSR markers were mapped on each of the three linkage maps. A reference map was developed, with 175 loci and 22 linkage groups, covering a total distance of 885.4 cM. The marker order was in general collinear to the A genome map of Moretzsohn et al. (2005).

Another intraspecific map for peanut was developed using a RIL population composed of 318 F_8/F_9 plants (Varshney et al. 2009a). Corroborating the known low genetic variation of peanut, only 135 microsatellite markers, of the 1,145 screened, mapped in 22 linkage groups spanning 1,270.5 cM. This map enabled the mapping of QTLs controlling drought tolerance-related traits as well as establishing relationships with diploid A genome of groundnut and model legume genome species.

In 2012, several moderately saturated maps and two proposed reference maps were published. Wang et al. (2012) reported a linkage map, based on an F_2

population of 94 individuals derived from *A. hypogaea* subsp. *hypogaea* × *A. hypogaea* subsp. *fastigiata* cross. The map consisted of 318 loci, mostly SSRs, onto 21 linkage groups covering a total distance of 1,674.4 cM. In this study, resistance gene homolog (RGH)-containing BAC clones were sequenced to develop SSR markers. Two of these markers were mapped into two different linkage groups, anchoring one RGH-BAC contig and one singleton, which can facilitate marker-assisted selection for disease resistance breeding and map-based cloning of resistance genes. Shirasawa et al. (2012a) developed a great number of microsatellite and transposon markers by in silico analysis and published the most saturated individual map for *Arachis* to date. A total of 1,114 markers were mapped into 21 linkage groups covering 2,166.4 cM based on an F_2 population ($n = 94$) derived from an *A. hypogaea* subsp. *hypogaea* × *A. hypogaea* subsp. *fastigiata* cross. The authors also published an intra-subspecific *A. hypogaea* subsp. *hypogaea* × *A. hypogaea* subsp. *hypogaea* map, with 326 markers and 19 linkage groups, spanning 1,332.9 cM.

An integrated map was constructed from two RIL populations (Qin et al. 2012). This map contained 324 markers covering 1,352.1 cM with 21 linkage groups. The translocation event that seems to have occurred between linkage groups A7 and A8 or B7 and B8 was also evident in comparison of this map to previously published diploid and tetraploid maps (Moretzsohn et al. 2005, 2009; Fonceka et al. 2009).

Two reference consensus linkage maps were published very recently for peanut (Gautami et al. 2012; Shirasawa et al. 2013). The first map (Gautami et al. 2012) was based on ten intraspecific RILs and one interspecific backcross population and comprised 895 microsatellite markers. Linkage groups were identified and named by comparisons with the diploid maps previously published for the A and B genomes of *Arachis* (Moretzsohn et al. 2005, 2009; Leal-Bertioli et al. 2009). The reference map was divided into 20 cM long 203 BINs having microsatellite markers with known polymorphism information content (PIC) values. The second map (Shirasawa et al. 2013) was based on three wild-derived RIL populations, integrated with another 13 maps already published and with sequences of other legume species. All this information will be useful for selecting highly polymorphic and uniformly distributed markers for further genetic studies and marker-assisted selection in peanut.

12.5 Achievements

12.5.1 The Different Routes for Interspecific Population Development: How to Access Wild Gene Reservoir in the Context of Ploidy Reproductive Barrier

One of the main constraints to hybridisation between cultivated peanut *A. hypogaea* and its wild relatives is the reproductive barrier caused by the difference in ploidy level. In order to access and use the large genetic diversity available in diploid species in a tetraploid context, one has to turn to bridging strategies allowing the crossability of wild species and the cultivated. Four different routes have been

described by Simpson (2001): the hexaploid pathway, two different diploid/tetraploid pathways and genetic engineering-based methods.

The hexaploid pathway consists in the direct cross of a given diploid species with *A. hypogaea*. The resulting hybrid is triploid and sterile and can be doubled to the hexaploid level after treatment by colchicine. In general, the hexaploid is cytologically unstable, and the tetraploid state needs to be recovered following chromosome loss through successive selfing or backcrossing to *A. hypogaea*.

The first diploid/tetraploid route consists of reconstructing a tetraploid from a two- or a multiple-way cross of different *Arachis* diploid species having different genome types. The hybrid is treated by colchicine to double the chromosome number. The resulting allotetraploid is cross compatible with cultivated peanut and can be used as a parent for an introgression programme. Alternatively, in the second diploid/tetraploid route, a single *Arachis* diploid species representative or hybrids between diploid species having the same genome can be colchicine doubled to form an autotetraploid that is cross compatible with cultivated peanut. Autotetraploids have however been described as weak plants and crossing with *A. hypogaea* reported as difficult (Holbrook and Stalker 2003).

As a fourth strategy, transformation technologies can be used to access genes from species of the tertiary gene pools or from outside the genus.

12.5.2 Wild Introgressions in Peanut: A Historical View

Several long-term programmes have been conducted to introgress valuable genes from wild *Arachis* species into cultivated peanut since the first interspecific hybridisations realised by Krapovickas and Gregory in the 1940s.

Using the hexaploid pathway, Stalker et al. (1979) generated a tetraploid interspecific population from a cross produced earlier by Smartt and Gregory (1967) between a cultivated peanut line (PI 261942-3), collected from Paraguay, and the A genome diploid species *A. cardenasii* (10017 GKP, PI 262141). The population was obtained after five generations of selfing from the colchicine-doubled triploid hybrid that allowed to recover tetraploid individuals with 40 chromosomes. The phenotypic characterisation of this population allowed the identification and selection of several lines for higher yield, resistance to leaf spots (*Cercospora arachidicola* and *Cercosporidium personatum*) as well as resistance to several insects. Using the ten highest yielding families of this population, a recurrent selection programme was conducted (Guok et al. 1986) and resulted in a significant increase in fruit yield and kernel yield components after two cycles of recurrent selection, providing an early evidence that favourable alleles for grain production can be gained from a wild *Arachis* diploid species. From the same population, several hybrid selections were identified as having significantly higher level of resistance to early leaf spot (Stalker 1984) than the most resistant cultivar evaluated at the same time. However, these hybrid derivatives had poor agronomic performance and could not be used directly as improved variety.

With the development of molecular markers, the same group of researchers characterised a set of lines from the same interspecific population with RFLP and RAPD markers (Garcia et al. 1995). Based on an existing RFLP linkage map (Halward et al. 1993), the analysis revealed the distribution of introgressed segments from *A. cardenasii* in the cultivated genetic background attesting of recombination events between the diploid genome and both genomes of the cultivated (88 % of the *A. cardenasii* introgression events were located in the A genome and 12 % were located in the B genome). One of the lines, identified as resistant to nematode (*Meloidogyne arenaria*), was crossed again to the cultivated parent to generate a segregating population from which two linked dominant resistance genes could be identified and designated as *Mae*, a dominant gene restricting egg number, and *Mag*, a dominant gene restricting galling. Using bulked segregant analysis (BSA), one RAPD marker was linked at 10 and 14 cM from *Mag* and *Mae*, respectively (Garcia et al. 1996). Two root-knot nematode-resistant varieties have been released following this research (Stalker et al. 2002a). In addition, late leaf spot resistance lines deriving from the same population were also registered (Stalker and Beute 1993; Stalker et al. 2002b) as well as insect resistant lines (Stalker and Lynch 2002).

More recently, the variety GPBD4 has been developed through pedigree selection from the cross between KRG1, an early maturing line from Argentina, and ICGV 86855 (Gowda et al. 2002), also referred to as CS16 (Vishnuvardhan et al. 2011), an interspecific derivative from *A. cardenasii*. GPBD4 is resistant to late leaf spot and rust and has spread over large area in the state of Karnataka in south of India (Gowda et al. 2002). A QTL mapping study involving a RIL population from the cross between GPBD4 and TAG24 allowed the identification of late leaf spot and rust resistance QTLs that could be related to early introgressions from *A. cardenasii* into the cultivated genome (Khedikar et al. 2010). Another case of utilisation of *A. cardenasii* was the development of a foliar disease-resistant variety using the hexaploid pathway from a primary cross between the variety CO1 and *A. cardenasii*. After three successive backcrosses with the cultivated parent and four generations of selfing, the variety VG9514 was selected (Varman 1999), and it showed good resistance to rust and late leaf spot. The rust resistance QTL identified by Khedikar et al. (2010) from GPBD4 was confirmed in a similar RIL population involving VG9514 and TAG24 as parents (Mondal et al. 2012).

Following the work with *A. cardenasii*, another landmark in the use of wild species occurred with the use of the tetraploid route. Simpson et al. (1993) created the first amphidiploid from a three-way cross between *A. cardenasii* and *A. diogoi* on the A genome side and *A. batizocoi* on the B genome side. The AB sterile hybrid was treated with colchicine to produce TxAG-6, a fertile amphidiploid that has been at the root of major genetic studies and breeding applications. The first tetraploid RFLP-based genetic map was constructed from an interspecific BC_1 population involving TxAG-6 as donor parent into the cultivated background of Florunner (Burow et al. 2001). The genetic map obtained was the first nearly saturated tetraploid map and allowed the genome-wide analysis of the transmission of chromatin between wild and cultivated species attesting of a similar recombination pattern as chromosome pairing reported for *A. hypogaea*. The RFLP nature of the markers

used in this study also made it possible to analyse the synteny conservation and colinearity between the two subgenomes of *A. hypogaea* showing a globally high level of conservation and some chromosomal rearrangements. TxAG-6 and TxAG-7, a backcross derivative (BC_1) of TxAG-6 with the variety Florunner, were released for their breeding potential for resistance to root-knot nematode and leaf spot (Simpson et al. 1993). TxAG-7 was further used as parent of a backcross population (BC_4F_2) from which genetic markers linked to root-knot nematode have been identified by bulk segregant analysis. This single gene, identified in *A. cardenasii* and transferred to peanut through TxAG-6, is currently the only dominant root-knot nematode resistance gene deployed in modern cultivars (Holbrook et al. 2008). By comparative genetic mapping in diploid and tetraploid peanut populations, this gene, called *Rma*, was found to have been introduced in a chromosome segment spanning one-third to one-half of chromosome 9A (Nagy et al. 2010). In the latter study, numerous codominant markers were identified for finer mapping of *Rma* and for marker-assisted selection for nematode resistance, by using two tetraploid RIL populations of *A. hypogaea* and an intraspecific F_2 diploid population from a cross between two *A. duranensis* accessions. Initially, two varieties were released from backcross derivatives of TxAG6 with Florunner: COAN and NemaTAM (Simpson and Starr 2001; Simpson et al. 2003). NemaTAM was almost immune to root-knot nematode but sensitive to tomato spotted wilt tospovirus (TSWV). Further improved varieties were developed starting either from NemaTAM or COAN and using either conventional methods or marker-assisted selection to develop varieties combining resistance to nematode and TSWV, like Tifguard (Timper et al. 2008) or Tifguard high O/L (Holbrook et al. 2011).

Another route, involving in vitro techniques, has also been described that allowed to tap wild species from tertiary gene pool of *Arachis* genus. Hybrids have been obtained between *A. hypogaea* and two diploid wild species from section *Procumbentes*, *A. chiquitana* and *A. kretschmeri* (Mallikarjuna and Hoisington 2009; Mallikarjuna and Tandra 2006). *Arachis glabrata* from section *Rhizomatosae* has also been successfully crossed with *A. hypogaea* (Mallikarjuna and Sastri 2002). The method includes embryo rescue from immature pods resulting from the interspecific cross between *A. hypogaea* and the diploid wild. To recover tetraploid state the authors benefited from numerically unreduced gametes or 2*n* pollen that are produced at low frequency from F_1 hybrids (Mallikarjuna and Tandra 2006). The same authors have also used the tetraploid route to produce a collection of 17 new allotetraploids and autotetraploids between different species of the secondary gene pool (Mallikarjuna et al. 2011). The different diploid species involved were *A. batizocoi*, *A. cardenasii*, *A. diogoi*, *A. duranensis*, *A. hoehnei*, *A. ipaënsis*, *A. kempff-mercadoi*, *A. magna*, *A. stenosperma* and *A. valida*. One allotetraploid (ISATGR 1212) and its reciprocal form (ISATGR-40A) had the same genomic composition of *A. hypogaea* originating from the cross of *A. duranensis* with *A. ipaënsis*. This collection of synthetics representing a wide coverage of diversity of the secondary gene pool of *Arachis* are a valuable resource for introgression of positive wild alleles into cultivated gene pool, and the crossability with cultivated peanut has been analysed for five of them (Mallikarjuna et al. 2012b). The use of these synthetics as

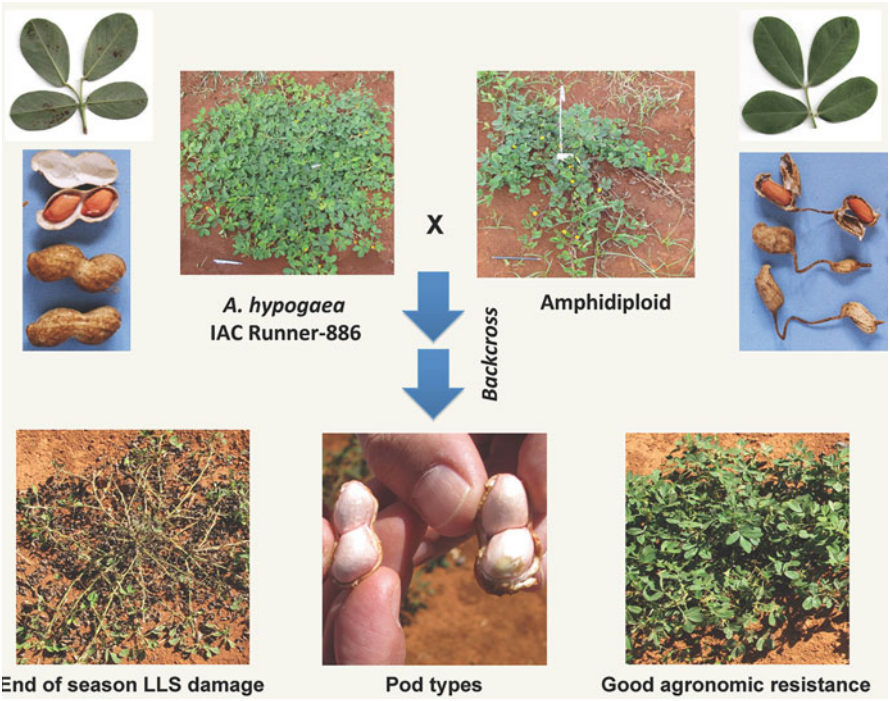

Fig. 12.2 Introgression of resistance for late leaf spot into the cultivated variety Runner IAC 886. After one backcross and field selection of backcross, lines combining resistance to LLS and good agronomic performance were selected

progenitors to conduct introgression programmes in cultivated peanut is in progress in breeding programmes in Senegal and in India.

Also using the tetraploid route, a synthetic amphidiploid has been developed in Brazil from the proposed ancestors of cultivated peanut (*A. ipaënsis* and *A. duranensis*) (Fávero et al. 2006). This amphidiploid donor has been crossed with Runner IAC 886 (a selection of Florunner), and 12 lines that combine agronomically adapted phenotype with resistance to late leaf spot have been selected using a combination of genotyping and phenotyping (Fig. 12.2) (Leal-Bertioli et al. 2010; Leal-Bertioli SCM, Moretzsohn MC, Guimaraes PM, Godoy I and Bertioli DJ unpublished results).

An introgression programme has been conducted using the same amphidiploid and Fleur 11, a popular variety from Senegal, as cultivated recipient. The whole process involved the construction of an interspecific SSR genetic map at the BC_1F_1 generation (Fonceka et al. 2009), followed by a large marker-assisted backcross scheme to monitor wild introgression distribution in the genome of the cultivated parent at each generation. The programme was conducted up to the BC_4F_3 generation to produce a set of chromosome segment substitution lines (CSSLs) that globally incorporate the whole genome of the wild ancestors as overlapping segments

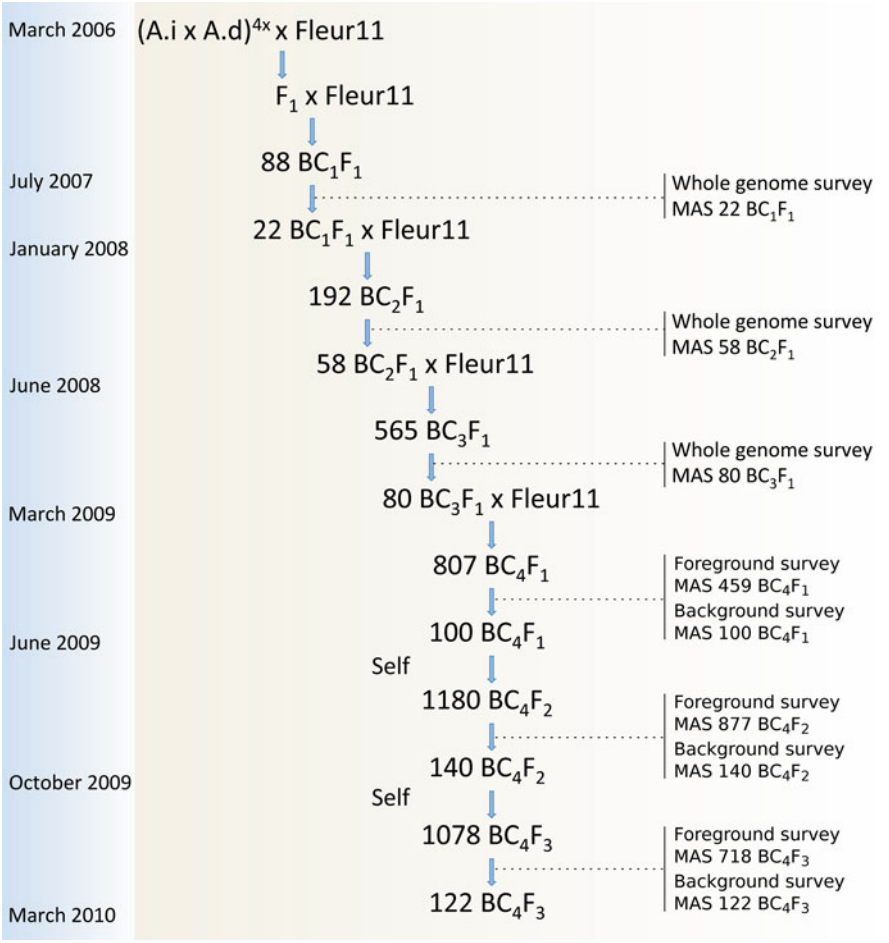

Fig. 12.3 Breeding scheme followed for the development of the CSSL population (Fonceka et al. 2012b)

introgressed in the recipient cultivar. The whole programme that represents seven generations was conducted over a period of 4 years (Fig. 12.3) thanks to off-season generations and the ability to successfully genotype progenies as part of the breeding process during the short time frame between seedling and flowering.

As part of this introgression programme, an advanced backcross (AB-QTL) population was derived from the plants that were not selected to be advanced to CSSLs at the BC_2F_1 generation (Fonceka et al. 2012a). The population was composed of a mix of BC_3F_1 and BC_2F_2 individuals that were allowed to self-pollinate to produce BC_3F_2 and BC_2F_3 families used for the phenotyping and QTL detection. Several traits concerning days to flowering, plant architecture, pod and seed morphology and yield components were analysed in this population leading to the identification of 95 QTLs in two different water regimes. As a first conclusion, it could be shown

that wild alleles contributed positive variation to many valuable agronomic traits such as flowering precocity, seed and pod number per plant, length and size as well as pod maturity. About half of the positive QTL effects were associated to the allele of the amphidiploid parent. In some cases, these QTLs, such as QTLs for seed length on chromosome a09, were associated to undesirable morphological traits like pod constriction or pod beak, probably requiring further backcrosses to reduce linkage drag. However, in several cases favourable wild QTLs had no detrimental association and could be directly used to improve the cultivated variety: QTLs for pod number and weight on chromosome a01, QTLs for seed number, total biomass and stress tolerance indices on chromosome a05 and QTLs for seed diameter on chromosome b06. Moreover, the comparison of QTLs obtained under well-watered and water-limited conditions revealed that QTLs for stress tolerance indices for pod and seed numbers with favourable alleles attributed to the wild parents could be involved in the trade-off between maintaining large-sized seed and producing more seeds under water stress. In addition, QTL clusters related to domestication syndrome, i.e. involved in plant and pod morphology as well as pod and seed size, were also mapped in the same study. QTLs that greatly affected pod and seed size appeared to be clustered in three genomic regions while those affecting the plant and pod morphology were dispersed across the genome. It was proposed from these findings that the main focus of human selection at the incipient stage of domestication could have been concentrated on pod and seed size, given the primitive growth habits and constriction depths that still exist in peanut cultivated species (Fonceka et al. 2012a).

The final CSSL population (Fonceka et al. 2012b) was composed of 122 lines offering a wide coverage of the peanut genome especially in the context of the large peanut genome size (c. 2,800 Mb/1C and 20 linkage groups) with target wild chromosome segments of 39.2 cM on average. Most of the CSSLs (62 %) contained a single wild fragment in a homogeneous cultivated genetic background (Fig. 12.4). For the lines that contained more than one fragment, additional backcrossing efforts are ongoing for deriving lines harbouring a unique wild chromosome segment. Using simple high-heritability traits like plant growth habit or pod constriction as a proof of concept, the value of the CSSL population could be illustrated. For example, an introgression line harbouring a single wild donor fragment corresponding to the location of a QTL for pod constriction identified in the AB-QTL population showed deeply constricted pods as compared to the moderate constriction observed with the cultivated parent, confirming the QTL previously identified. Similarly, two lines harbouring single overlapping donor fragments in the region of a QTL for plant growth habit showed contrasting phenotypes, allowing to confirm and refine the position of this QTL (Fig. 12.5).

12.6 Conclusion and Further Prospects

As described in the previous sections, wild introgressions in peanut have now been carried out for many years, particularly because of the specific interest this approach represents for the improvement of this crop. However, in spite of those important

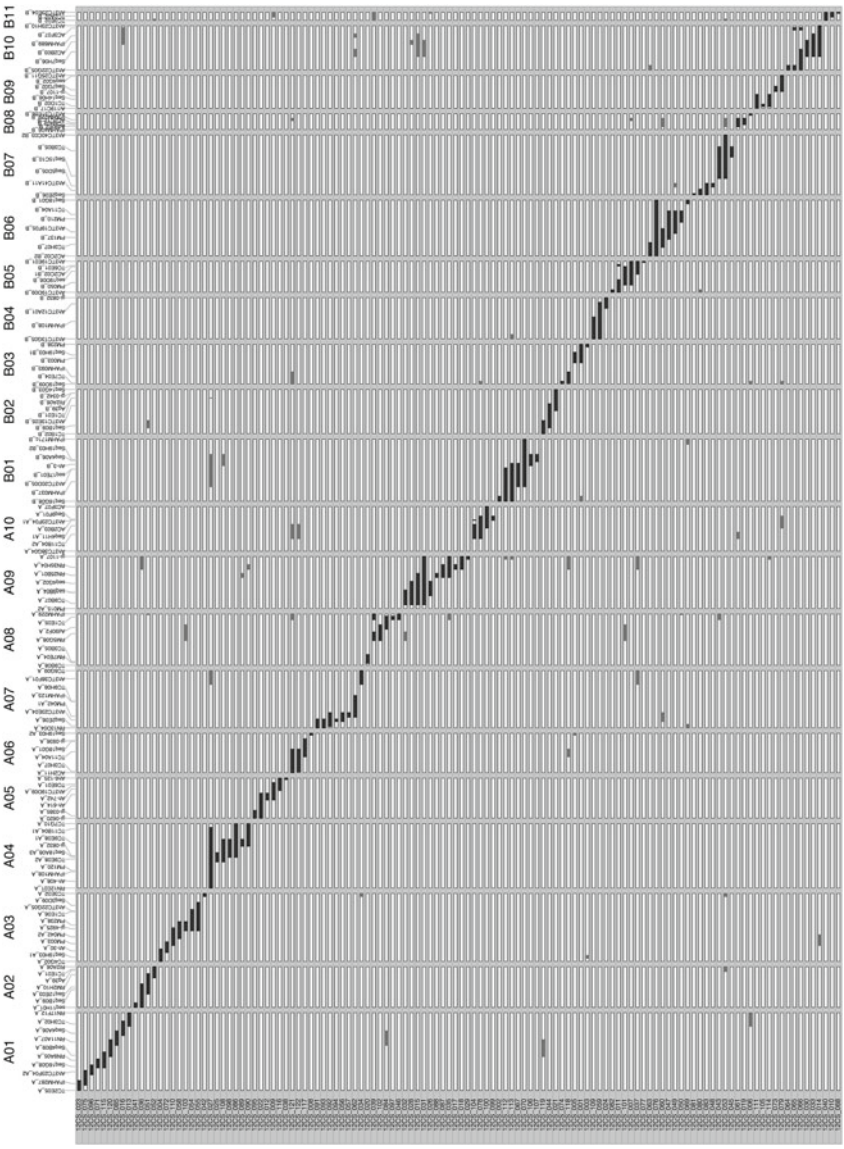

Fig. 12.4 Graphical genotypes of the 122 CSSLs (reproduced from Fonceka et al. 2012b)

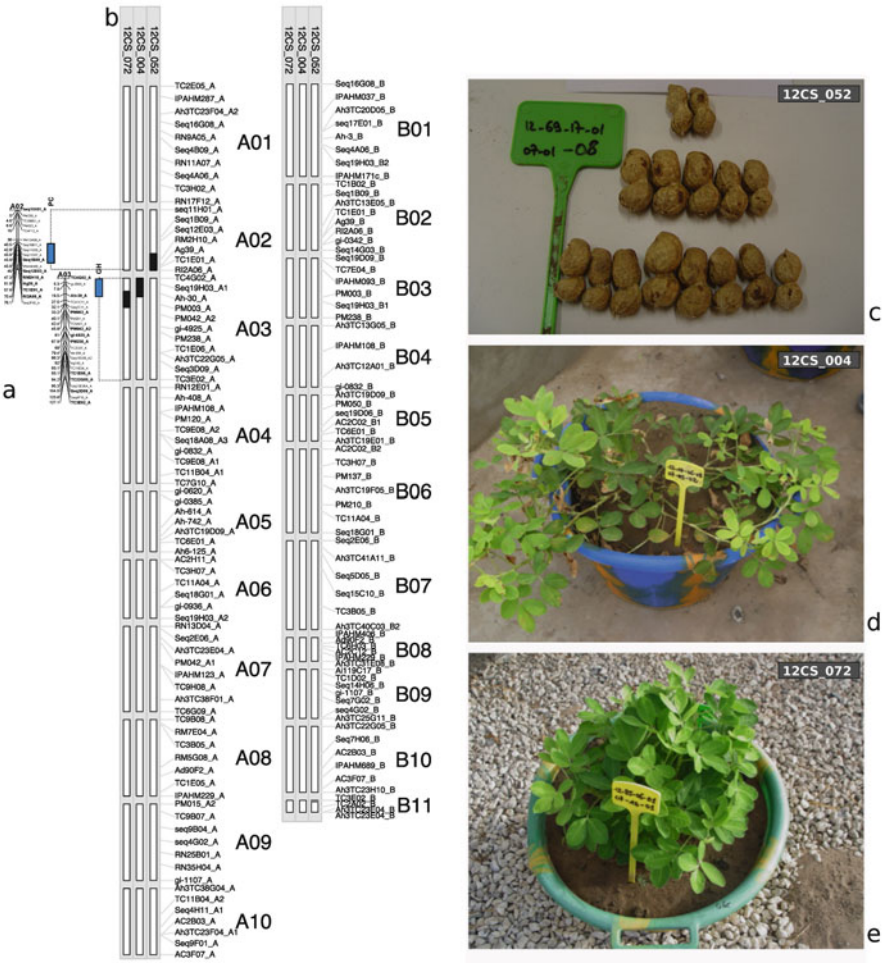

Fig. 12.5 Relation between introgression and phenotype for pod constriction and plant growth habit for three CSSL lines. (**a**) QTLs detected in AB-QTL population for pod constriction (PC) on linkage group a02 and for plant growth habit (GH) on linkage group a03. (**b**) Graphical genotype of three CSSL lines corresponding to the same QTLs. (**c**) Phenotype of the CSSL line 12CS_052 for pod constriction. (**d**) Phenotype of the CSSL line 12CS_004 for plant growth habit. (**e**) Phenotype of the CSSL line 12CS_072 for plant growth habit

achievements, and mainly due to the limitations of the plant itself in terms of crossability, multiplication rate, and, until recently, lack of appropriate molecular tools, the extent of utilisation of the useful allele reservoir of the wild species and its impact on peanut breeding have been limited. For now, successful introgression of wild genes into cultivated peanut concerns few wild species. *Arachis cardenasii* has probably been one of the most used sources of useful genes to date even if crosses involving other species have also been used. The recent use of the two most probable ancestors of peanut *A. duranensis* and *A. ipaënsis* in a systematic

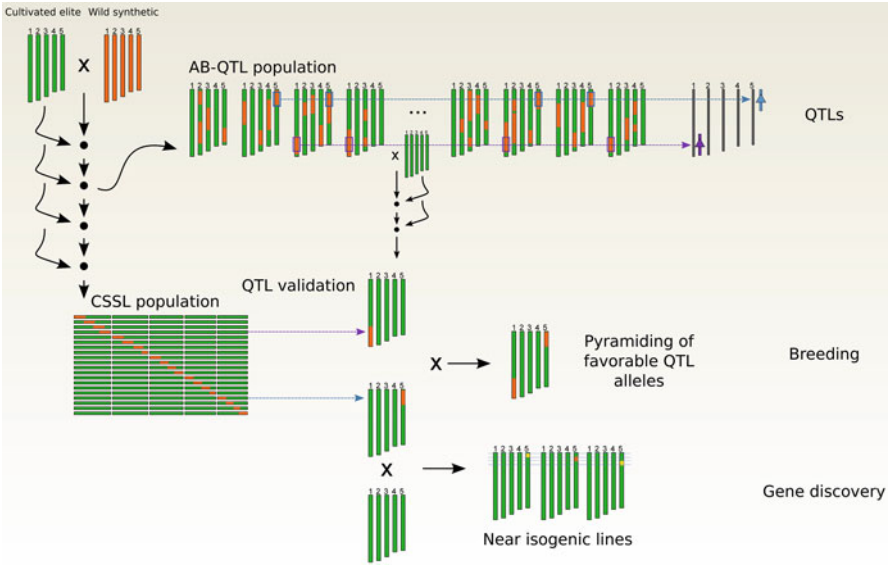

Fig. 12.6 A general strategy for harnessing the potential of peanut wild relatives using AB-QTL and CSSL populations

introgression programme opens the way for extensive and detailed characterisation of genetic determinism and wild alleles' effects on a wide range of traits. However this resource only involves two representatives of two wild species, and the potential in generalising introgression programmes to other accessions of the same species and to other species of the secondary gene pool is immense.

A general strategy to harness this potential is proposed in Fig. 12.6. One of the major lessons learned through the utilisation of wild species in crop improvement is that cryptic valuable alleles can be found in wilds that can be identified and characterised only once it is incorporated in a cultivated genetic background. As proposed by Tanksley and Nelson (1996), advanced backcross QTL analysis has the potential to simultaneously identify wild QTL alleles while delivering genetic material directly applicable to breeding. One extension of this approach is to construct a CSSL library through more backcross generations and a higher control of introgression sizes and distribution. However, this last approach requires a large investment in terms of crossing and genotyping and cannot be generalised to a wide range of wild donors. Nevertheless, single-fragment introgression lines can be derived from AB-QTL populations on a QTL-by-QTL basis. Such lines, once the wild effect has been validated and characterised, can be crossed to accumulate favourable alleles for different traits toward variety release. They can also be used for deriving near-isogenic lines through further backcrossing providing experimental material for map-based gene cloning. The implementation of this strategy requires a close integration of genotyping in the breeding process, which can now be widely achieved, thanks to the development of molecular markers and genetic maps in peanut and the availability of genotyping platforms offering fast and reliable genotyping services.

Such a large-scale generalised introgression programme would require a concerted effort of the peanut international community. Taking into account the number of wild accessions available in gene banks, a rational sampling of target donors would have to be achieved, regarding peanut breeding objectives, based on the characterisation knowledge base that already exists on wild diseases resistance, ecological adaptation, fertility and crossability. A concerted strategy would also be required on the choice of cultivated backgrounds for introgression. A first option (the one that has been used in tomato) would be to use a common cultivated background allowing the direct comparison of the effects of introgressions from different species and the optimisation of the introgression effort among the community. However, the disadvantage of this option resides in the fact that a common cultivated background may not be adapted to some of the target environments compromising the potential of direct breeding application of introgressed material. As an alternative, the choice of specific targeted elite cultivars by improving wild crosses seems to offer a better tradeoff between breeding opportunities and genetic analysis.

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Chapter 13
Sunflower

Yalcin Kaya

Abstract Sunflower ($2n = 17$) belongs to the genus *Helianthus* (Asteraceae) which consists of 52 species and 19 subspecies with 14 annual and 38 perennial species. Wild *Helianthus* species display large morphological variations and have valuable genes for several traits including high seed and oil yield, oil quality, and resistance to abiotic and biotic stresses. It is easier to cross the annual species than the perennial ones because perennial species are much more diverse and their genomes display different ploidy levels. The difficulty of crossing in wild perennial *Helianthus* species can be overcome by the novel molecular breeding techniques which have made these species available for breeding purposes and alien gene transfer into cultivated background. The beneficial genes from wild species have broadened the genetic base of cultivated sunflower providing valuable sources for many agronomic traits. Consequently, significant progress has been made in transferring resistance to new races of downy mildew, rust, broomrape, and other diseases such as *Phoma*, *Phomopsis* and *Sclerotinia*. Sources for cytoplasmic male sterility and fertility restoration genes have been identified besides new genes for improving oil quality, herbicide resistance, and salt tolerance. However, only a small portion of the available genetic diversity of the wild *Helianthus* species has been utilized until now, and therefore quest for search of more alien genes is likely to continue in sunflower.

Keywords Alien gene transfer • CMS • *Helianthus* • Hybridization • Introgression • Oil quality • Sunflower

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13.1 Introduction

Sunflower is one of the widely grown summer crops in the world, and hybrids are commonly used in its production. However, its seed yield has not increased like corn in the hybrids, mainly due to wide use of only a single cytoplasmic male sterility (CMS) source PET1 and a few fertility restoration genes that lead to decreased heterosis worldwide in the hybrid production. In addition, limited genetic variability of cultivated sunflower puts its cultivation in a vulnerable position, and many diseases and pests attack this crop easily (Škorić 2012).

The genus of *Helianthus* consists of 51 species with annual and perennial forms and is a much-diversified genus in various environments. Mandel et al. (2011) mentioned higher level of genetic diversity in wild *H. annuus* populations based on SSR markers. One of the promising ways to increase genetic variability in sunflower is interspecific hybridization utilizing rich variability of wild relatives of *Helianthus*. Interspecific hybridization could play a key role to increase genetic variability and widen the genetic base of cultivated sunflower for developing highly productive hybrids possessing adaptability levels greater than the existing ones. In addition, wild species, for example, *H. mollis*, *H. divaricatus*, *H. californicus*, *H. floridanus*, and *H. maximiliani*, could be used to alter plant structure by developing new genotypes with shorter internodes and much shorter petioles because cultivated sunflowers have larger plant diameters (Kaya et al. 2012). This could help in accommodating more number of plants per unit area, thereby increasing productivity of the hybrids. Similarly, the competitive ability of root system also needs to be improved, both to adapt to marginal environments and also to increase nutrition and water uptake in input limited areas (Seiler 2008).

Sunflower improvement by conventional breeding is severely constrained by the availability of a rather limited gene pool in practical sense owing to natural incompatibilities, even between related species, and also by the time-consuming process of most of the breeding programs. On the other hand, sunflower production continues to face challenges from both abiotic and biotic factors as well as rapidly changing market need and shifting production from areas of high productivity to marginal areas with lower yield potential (Seiler 2011). Therefore, to obtain useful genes from wild *Helianthus* species, novel biotechnological tools are employed which complement the traditional breeding methods to overcome the existing problems in interspecific hybridization and alien gene transfer (Škorić 2012; Seiler 2012). A number of alien genes from wild and exotic germplasm for resistance to many pathogens and herbicides and also genes for oil quality and other agronomic traits have been introduced from other *Helianthus* species to the cultivated types, and such genotypes are being used commonly and successfully (Seiler and Marek 2011). However, further molecular improvement of sunflower could be limited mostly by the lack of our knowledge about, and access to, important and useful genes (e.g., those controlling multigenic traits like yield and resistance to biotic and abiotic stresses).

13.2 Wild Genetic Resources

Wild sunflower species are adapted to a wide range of habitats and possess considerable variability for many agronomically important traits including resistant to insect pests and disease pathogens. Based on the numerical taxonomy, *Helianthus* genus (Table 13.1) is divided into four sections (Schilling and Heiser 1981; Schilling 2001; Fernandez et al. 2009). This genus has a basic chromosome number of $n=17$ and contains diploid ($2n=2x=34$), tetraploid ($2n=4x=68$), and hexaploid ($2n=6x=102$) species. The 14 annual species are all diploid, and the 38 perennial species include 25 diploid, 3 tetraploid, 7 hexaploid, and 3 mixaploid species. *H. ciliaris* and *H. strumosus* have both tetraploid and hexaploid forms, while *H. decapetalus* and *H. smithii* contain diploid and tetraploid forms (Seiler and Marek 2011; Seiler 2012).

Helianthus genus originated and was domesticated in North America, and the wild species found in other continents are almost certainly introductions. Wild forms of *Helianthus annuus* exist as common weeds in many parts of the USA, while other *Helianthus* species have varying distributions in the USA, Canada, and Mexico. In spite of this, initial crop developmental efforts for genetic improvement of sunflower as an oilseed crop were only undertaken in Russia in the nineteenth and early twentieth centuries, and genetic improvement was mostly focused on development of open pollinated cultivars. However, after the discovery of CMS and fertility restorer systems in the 1970s, hybrid development was pursued and wild *Helianthus*, *H. annuus*, or others as source of variability were used for breeding of cultivated varieties (Vear 2011; Škorić 2012; Seiler 2012).

13.3 Collection, Evaluation, and Maintenance of Wild Genetic Resources

The wild species of *Helianthus* have been used to some extent; however, the tremendous amount of genetic diversity available in these resources is yet to be exploited, and it requires understanding of the amount and distribution of the genetic variation. Nooryazdan et al. (2010) investigated the patterns of distribution of morphological variation for 13 quantitative characters in 77 accessions of wild sunflower and detected wide variation on seed weight, petiole and lateral branch length, plant height, head diameter, and sowing–flowering duration among accessions. Therefore it is necessary to collect, maintain, characterize, evaluate, and utilize the wild germplasm to fulfil the growing needs for additional genetic variability.

It has been observed that the loss of variation in crops, i.e., genetic erosion of cultivated diversity, occurred initially due to the replacement of landraces by modern cultivars and subsequently by modern breeding practices. Unfortunately, the survival of some sunflower species under long-term preservation has not proven to be

Table 13.1 Sections, series, and subspecies for the *Helianthus* with chromosome numbers[a]

Section	Series	Species	Subspecies	(2*n*)
I. Agrestis		*agrestis*		34
II. Annuui		*niveus*	*niveus*, *tephrodes*, *canescens*	34
		debilis	*debilis*, *vestitus*, *tardilorus*, *silvestris*, *cucumerifolius*	34
		praecox	*praecox*, *runyonii*, *hirtus*	34
		petiolaris	*petiolaris*, *fallax*	34
		neglectus, *annuus*, *argophyllus*, *bolanderi*, *anomalus*, *paradoxus*		34
III. Ciliares	1. *Pumili*	*gracilenthus*, *pumilus*, *cusickii*		34
	2. *Ciliares*	*arizonensis*, *laciniatus*		34
		ciliaris		68
IV. Atrorubentes	1. *Divaricati*	*mollis*, *divaricatus*, *decapetalus*		34
		occidentalis	*occidentalis*, *plantagineus*	34
		hirsutus, *strumosus*		68
		eggertii, *tuberosus*, *strumosus*		102
		rigidus	*rigidus*, *subrhomboideus*	102
	2. *Gigantei*	*giganteus*, *grosseserratus*		34
		nuttallii	*nuttallii*, *parishii*, *rydbergii*	34
		maximiliani, *salicifolius*		34
		resinosus, *schweinitzii*, *californicus*		102
	3. *Microcephali*	*microcephalus*, *glaucophyllus*, *smithii*, *longifolius*		34
		laevigatus		68
	4. *Angustifolius*	*angustifolius*, *simulans*, *floridanus*		34
	5. *Atrorubentes*	*silphioides*, *atrorubens*, *heterophyllus*, *radula*, *carnosus*		34

[a]Jan and Seiler (2007), Fernandez et al. (2009), Breton et al. (2010), Seiler and Jan (2010), Vear (2011)

promising, and some species are already rare and endangered, while *H. nuttallii* T. and G. ssp. *parishii* (A. Gray) Heiser have probably become extinct (Vear 2011; Seiler and Marek 2011; Seiler 2012). Hence efforts have been made in collection and maintenance of the wild species for sustainable genetic improvement in sunflower. A total of 39,380 accessions of sunflower have been reported from 92

institutions (Vear 2011). The wild *Helianthus* species collection of the USDA-ARS, presently located at Ames, IA, is the most comprehensive collection in the world containing 2,163 accessions, about two-thirds of which are annual species (Seiler and Marek 2011). Other collections are at INRA, Montpellier, France, with more than 600 accessions of 45 wild sunflower species (Vear 2011); Institute of Field and Vegetable Crops, Novi Sad, Serbia, 39 wild species with 447 accessions (Atlagić et al. 2006); and the Dobroudja Agricultural Institute (DAI) at General Toshevo, Bulgaria, where 428 accessions represent 37 species of *Helianthus* (Christov 2008). Other wild collections exist in the Instituto de Agricultura Sostenible, Cordoba, Spain, maintaining 44 annual and perennial accessions of *Helianthus* (Ruso et al. 1996), and 36 wild *Helianthus* species exist in NPGS, the Directorate of Oilseed Research, Hyderabad, India (Mulpuri 2006). Another one is in Veidelevka Institute of Sunflower, Russia, which has a wild sunflower collection containing 8 annual species (208 accessions) and 27 perennial species (227 accessions) (Tavoljanski et al. 2002, 2004). N. I. Vavilov Research Institute (VIR), St. Petersburg, Russia, also maintains 550 accessions of 29 species with 128 perennial accessions. Nevertheless, the present germplasm collection throughout the world contains only a portion of the available genetic variability in *Helianthus*. The economic contribution of the wild species to the cultivated sunflower industry in the USA has been estimated to range from $269.5 million per year to $384 million per year. At current crop prices, this would translate to close to one billion USD (Seiler and Marek 2011).

13.4 Distant Hybridization

Interspecific hybridization represents a complementary technique in sunflower breeding and has been successfully used to produce new avenues of genetic variability. In spite of all difficulties like differences in the ploidy levels ($2x$, $4x$, $6x$) and genetic incompatibilities, distant hybridization has been regarded as an accessible method to introduce alien genes from wild germplasm into sunflower cultivars. Interspecific hybridization has been used for achievement of objectives like improvement of resistance to abiotic stresses, higher oil content and protein quality, identification of new sources for cytoplasmic androsterility and pollen fertility restoration, and even selection of some agronomically useful traits (Sauca and Lazar 2011; Škorić 2012; Seiler 2012).

13.4.1 Crossability Potential

The crossability between cultivated and wild species is one of the important and easiest ways to transfer the alien genes in crop plants. During the past years, several studies have been conducted in order to assess the crossability potential between the cultivated and wild sunflower species (Georgieva-Todorova 1984; Christov

1991; Encheva et al. 1992; Quillet et al. 1995; Atlagić 1996; Seiler and Rieseberg 1997; Serieys 1999; Faure et al. 1998, 2002; Mulpuri 2006; Hristova-Cherbadzi and Christov 2008; Hristova-Cherbadzi 2009; Seiler 2011; Sauca and Lazar 2011). It has been observed that crosses between cultivated sunflower and annual species in *Helianthus* section have been produced without much difficulty except with *H. agrestis*. Nonetheless, the resulting progenies are more or less male sterile due to translocations (Quillet et al. 1995).

Crosses between sunflower and perennial species in *Atrorubens* section are much more difficult to perform except when the male is a polyploid, such as *H. tuberosus* (Vear 2011). The difficulty of crossability between diploid perennials and the cultivated sunflower has been mentioned in many studies (Georgieva-Todorova 1984; Atlagić 1994; Atlagić et al. 1995; Hristova-Cherbadzi and Christov 2008). Hristova-Cherbadzi et al. (2008, 2012) directly crossed the perennial hexaploid species *Helianthus pauciflorus* (*rigidus*) with cultivated *H. annuus* L. They observed that crossability rate was high and the F_1 hybrids strongly resembled the wild parent in most important biomorphological characters. Successful crossing between cultivated and wild sunflower may sometimes need in vitro procedures as well as cytogenetic analyses (meiosis and pollen viability). Hybrids between *H. mollis* and *H. maximiliani* as well as with cultivated sunflower were obtained successfully following the embryo culture method (Krauter et al. 1991). Chandler et al. (1986) found that all annuals were crossable with each other and also with the cultivated sunflower utilizing embryo rescue. The analyses of meiosis and pollen viability, which included all annuals and their F_1 interspecific hybrids, showed that the annuals differed in 0–6 translocations and 0–8 paracentric inversions. This substantiated that the basic chromosome number ($n = 17$) is not a single genome.

13.4.2 Crossability Barriers

Several crossability barriers such as cross-incompatibility, embryo abortion, embryonic and postembryonic interspecific and intergeneric incompatibility, sterility and reduced fertility in interspecific hybrids, and tendency of returning to parental genotypic combinations are encountered in distant hybridization in *Helianthus* (Seiler and Marek 2011). Poor crossability and frequent F_1 sterility in interspecific hybrids limit the usefulness of many wild *Helianthus* species. Abortion of the hybrid embryo is an important phenomenon that prevents interspecific hybridization.

The crossability rate and seed set are dependent not only on the number of chromosomes in the species but also on several other factors that influence zygote formation (Hristova-Cherbadzi 2009). Meiotic abnormalities and reduced pollen viability in F_1 hybrids between wild annual species and the cultivated sunflower have been reported by Georgieva-Todorova (1984), Whelan (1979), and Atlagić (1988, 1990). Chromosome structural differences result in differential genomic permeability. Selection against alien genes has the same effect, except that resistance to introgression appears in restricted genomic regions. Thus, barriers to introgression

among sunflower species include both chromosomal structural and genetic factors (Rieseberg et al. 1995). These factors reduce the levels of interspecific gene flow in *Helianthus* (Gross et al. 2003). To counter these barriers, suggested models are sib-crossing or selfing, in combination with backcrossing, which are more effective than backcrossing alone for introgression across linkage groups where recombination rates are low (Rieseberg et al. 1995, 2012).

Seeds were obtained in both directions of crossing, and higher success percentages and numbers of hybrid plants were obtained when cultivated sunflower was used as a female parent with the exception of the hybrids with the participation of all perennial tetraploid species such as *H. laetiflorus* and *H. pauciflorus* ssp. *rigidus*. The hybrids with the perennial species *H. nuttallii* was a leap in interspecific hybridization in sunflower because successful hybridization with this wild species had been reported for the first time (Hristova-Cherbadzi 2009).

13.4.3 Strategy to Overcome Crossability Barriers

Most of the wild annual species are responsive to crossing with cultivated sunflower, except for *H. agrestis*. However, the perennial diploids encounter great difficulties in crossing (Seiler and Marek 2011). Effective protocols have been developed to overcome the difficulties and allow gene transfer from wild species into cultivated sunflower (Table 13.2).

13.4.3.1 Embryo Rescue

The embryo rescue technique is most commonly used for overcoming the incompatibility between *H. annuus* and other alien wild species allowing to obtain a large number of interspecific hybrids (Georgieva-Todorova 1984; Bohorova et al. 1985; Krauter et al. 1991; Friedt 1992). This method has made it possible to investigate the possibilities of direct organogenesis as an approach to overcome interspecific and intergeneric incompatibility in sunflower hybridization (Encheva et al. 1992). Chandler and Beard (1983) developed a two-step embryo culture procedure that enabled them to produce 53 interspecific cross combinations without the endless efforts of pollination for seed production. However, this method was not successful for crosses between the difficult-to-cross perennial diploid and the cultivated sunflower. Jan and Fernandez-Martinez (2002) improved embryo rescue technique with colchicine treatment for chromosome doubling of F_1 plants to restore fertility and obtained successful interspecific hybridization of annual and perennial species.

Christov and Vassilevska-Ivanova (1999) successfully applied direct organogenesis method in immature F_1 hybrid embryos for the first time in sunflower for production of new forms from the intergeneric cross *Helianthus annuus* (cv. *Albena*)×*Verbesina helianthoides* (genus *Verbesina*). The embryo rescue procedure enhanced the level of partial hybridization, determined using RAPD or RFLP markers (Vear 2011).

Table 13.2 Methods to overcome crossability barriers in sunflower

Method	Cross combination	References
Reciprocal crosses	*H. mollis* × *H. orgyalis* *H. bolandery* × *H. annuus* *H. neglectus* × *H. annuus* *H. petiolaris* × *H. annuus* *H. divaricatus* × *H. annuus* *H. giganteus* × *H. annuus* *H. glaucophyllus* × *H. annuus* *H. divaricatus* × *H. annuus* *H. giganteus* × *H. annuus* *H. glaucophyllus* × *H. annuus* *H. maximiliani* × *H. annuus* *H. nuttallii* × *H. annuus* *H. occidentalis* × *H. annuus* *H. pumilus* × *H. annuus* *H. decapetalus* × *H. annuus* *H. hirsutus* × *H. annuus* *H. laevigatus* × *H. annuus* *H. ciliaris* × *H. annuus* *H. laetiflorus* × *H. annuus* *H. pauciflorus* ssp. *rigidus* × *H. annuus* *H. pauciflorus* ssp. *subrhomboideus* × *H. annuus* *H. debilis* × *H. annuus* *H. praecox* × *H. annuus* *H. petiolaris* × *H. annuus* *H. argophyllus* × *H. annuus* *H. argophyllus* × *H. petiolaris* *H. praecox* × *H. petiolaris*	Škorić et al. (1995), Faure et al. (1998, 2002), Mulpuri (2006), Jan and Seiler (2007), Hristova-Cherbadzi (2009), Seiler and Marek (2011), Sauca and Lazar (2011), Seiler and Jan (2010), Seiler (2011, 2012)
Growth regulators	*H. tuberosus* × *H. annuus*	Friedt (1992), Škorić et al. (1995), Seiler and Rieseberg (1997)
Protoplast fusion	*Helianthus giganteus* × *H. annuus* *H. tuberosus* × *H. annuus*	Krasnyanski and Menczel (1995), Seiler and Rieseberg (1997), Škorić et al. (1995)
Anther culture	*H. resinosus* × *H. annuus* *H. tuberosus* × *H. annuus*	Škorić et al. (1995), Nenova et al. (2000), Mulpuri (2006), Jan and Seiler (2007), Seiler (2011, 2012)
Embryo rescue	*H annuus* × *Verbesina helianthoides* *H. pauciflorus* × *H. annuus* *H. tuberosus* × *H. annuus* *H. hirsutus* × *H. annuus*	Chandler and Beard (1983), Georgieva-Todorova (1984), Krauter et al. (1991), Škorić et al. (1995), Atlagić (1996), Seiler and Rieseberg (1997), Sukno et al. (1999), Encheva et al. (1992)
Chromosome doubling by colchicine	*H. maximiliani* × *H. annuus* *H. nuttallii* × *H. annuus*	Jackson and Murray (1983), Jan (1988), Škorić et al. (1995), Seiler and Rieseberg (1997), Jan and Fernandez-Martinez (2002), Jan and Seiler (2007)
Use of bridge species	*H. maximiliani* × *H. annuus* *H. giganteus* × *H. annuus*	Whelan (1976), Whelan and Dorrell (1980), Seiler and Rieseberg (1997), Scascitelli et al. (2010)

13.4.3.2 Chromosome Doubling

Development of inbred lines in sunflower usually takes 6–8 years. However, completely homozygous lines can be obtained using dihaploidy within or less than a year, thus accelerating breeding process (Miller and Fick 1997). Chromosome doubling increased pollen grain size and sustainability of interspecific sunflower hybrids. The increased pollen grain size directly reflected chromosome doubling and provided a reliable criterion for classifying treated plants (Friedt 1992; Seiler and Rieseberg 1997). It restores fertility of amphiploids by providing an identical pairing partner for each chromosome. Although some promising results regarding anther culture were obtained, further research is needed to optimize haploid production in sunflower breeding, mainly owing to problems like low regeneration rate and genotype dependency (Nenova et al. 2000; Miladinovic et al. 2012).

Induced polyploidy from the hybrids *H. giganteus* × *H. microcephalus*, *H. microcephalus* × *H. giganteus*, and *H. maximiliani* × *H. decapetalus* (2*n*) was obtained with colchicine treatment. Similarly, induced tetraploids have also been obtained utilizing colchicine in *H. annuus* (Jan 1996). Jan (1988) reported a modified chromosome doubling technique using colchicine on 19 embryo-cultured wild × cultivated interspecific hybrids and its positive effect on a backcrossed seed set. In the same study, a 5-h colchicine treatment with 20 g/kg dimethyl sulfoxide in wild × cultivated cross resulted in a high frequency of chromosome doubling and the production of autotetraploid lines. The same colchicine treatment of interspecific F_1 hybrids also resulted in high frequencies of chromosome doubling and production of amphiploids in order to bypass the barriers and enable the transfer of desirable genes successfully (Jan and Fernandez-Martinez 2002). These amphiploids had restored fertility and provided additional genetic diversity for sunflower improvement and could be backcrossed with cultivated lines without using embryo culture. In general, it is expected that without chromosome doubling, the frequency of obtaining BC_1F_1 seeds will be generally low and frequency of weak BC_1F_1 plants will be high. However, with chromosome doubling, reduced pairing of chromosomes of *H. annuus* is expected with the chromosomes of wild *Helianthus* species due to preferential pairing of *H. annuus* chromosomes during meiosis (Jan and Seiler 2007).

13.4.3.3 Reciprocal Crossing

Reciprocal crossing offers additional possibilities for creating variability for the traits controlled by cytoplasmic genes. Such crosses could be used for finding new CMS sources derived from interspecific crosses (Hristova-Cherbadzi 2009). Sixty-two CMS sources have been identified from progenies of crosses made between wild *Helianthus* accessions and cultivated lines (Seiler and Marek 2011). Crossability between wild and cultivated accessions was tested on the basis of direct and reciprocal crossing. As the result, all annual and 14 perennial species have been crossed successfully with cultivated sunflower by the conventional hybridization method (Atlagić 2004; Atlagić et al. 2006, 2012).

Table 13.3 Species from genus *Helianthus* crossed with *H. annuus* (Hristova-Cherbadzi 2009)

Groups of species	Species
Annual species ($2n=34$)	*H. bolanderi*, *H. neglectus*, *H. petiolaris*
Perennial diploid species ($2n=34$)	*H. divaricatus*, *H. giganteus*, *H. glaucophyllus*, *H. maximiliani*, *H. nuttallii* ssp. *rydbergii*, *H. occidentalis* ssp. *plantagineus*, *H. pumilus*
Perennial tetraploid species ($2n=68$)	*H. decapetalus*, *H. hirsutus*, *H. laevigatus*
Perennial hexaploid species ($2n=102$)	*H. ciliaris*, *H. laetiflorus*, *H. pauciflorus* ssp. *rigidus*, *H. pauciflorus* ssp. *subrhomboideus*

It has been observed that crossing of the diploid species *H. occidentalis*, *H. maximiliani*, and *H. mollis* with cultivated species is difficult. However, Faure et al. (2002) were able to cross-cultivate sunflower with *H. mollis* following reciprocal crossing. All the plants were diploid, but reciprocal crosses led to different progenies with phenotypes that were predominantly similar to the female parent. Some examples of successful crosses from some wild species based on their chromosome numbers are given in Table 13.3.

13.4.3.4 Use of Bridge Species

Efficient use of chromosomal doubling techniques provided a means of F_1 fertility restoration and production of fertile amphiploids, which were used as a bridge species in alien gene transfer (Jan and Seiler 2007). For example, wild *H. annuus* has been used as an intermediate parent or bridge species to produce the first hybrids between the cultivated species and *H. giganteus* and *H. maximiliani* (Whelan 1978). Using embryo rescue and a colchicine treatment for chromosome doubling, interspecific amphiploids have been produced and used as bridge species to transfer genes for broomrape resistance from wild perennial *Helianthus* species into cultivated sunflower (Jan and Fernandez-Martinez 2002). Also the hexaploid perennial sunflower *H. tuberosus*, commonly known as Jerusalem artichoke, can be a useful bridge species because it crosses readily with many diploid annual and perennial species (Jan 1997). Similarly, Scascitelli et al. (2010) mentioned that longer history of intermittent contact between *H. debilis* and *H. annuus* and that *H. annuus* ssp. *texanus* could serve as a bridge for the transfer of alleles between its parental taxa.

13.4.3.5 Growth Hormones

Balanced concentration of growth regulators is important to regenerate interspecific hybrids through in vitro culture (Pugliesi et al. 1991, 1993). Growth regulators can also help to break seed dormancy, which is a major issue for using wild genetic resource efficiently in sunflower. Kantar et al. (2012) indicated that gibberellic acid was the best chemical treatment, initiating plant growth within 7–11 days in the

majority of the genotypes for artificially breaking dormancy in *H. tuberosus* and interspecific hybrids of *H. annuus*×*H. tuberosus*. Chandler and Jan (1985) indicated that low level of GA3 as a growth hormone increased germination up to 70 % in interspecific crosses as well as in wild populations, especially the perennial species.

13.4.3.6 Backcrossing

Traditionally, backcrossing is practised to introgress useful alien genes from wild relatives into breeding materials. For obtaining competitive inbred lines, at least three more generations of backcrossing are required, accompanied by a stringent selection in each generation for different desirable traits (Škorić 2012). While introgressing genes from wild gene pool, it is a common practice to resort to backcrossing. However, through backcrossing, it is difficult to obtain an optimized blend of genes from wild and cultivated types, and the progeny is more towards the cultivated forms. Obviously, the genotypes having most of their genetic background from cultivated species may fail to provide additional advantages and, hence, may have limited utility. On the contrary, restricting to the BC_1F_2 generation for selecting superior introgressions and their intercrossing may be more rewarding in getting lines having high heterogeneity and per se performance (Seiler 2012; Fick and Miller 1997).

Hristova-Cherbadzi (2009) used backcrossing in sunflower breeding followed by self-pollination, sib-pollination, and selection in different generations. Most of the new genotypes developed were resistant to some economically important fungal diseases and the parasite *Orobanche cumana* and had high combining ability and new plant architecture. On the other hand, while incorporating desirable traits from the wild relatives into the cultivated sunflower, some undesirable ones are also introduced (linkage drag). To overcome this problem, Atlagić et al. (2003) used backcrosses (F_1 interspecific hybrids×cultivated sunflower), although very often desirable traits are also lost in the process.

13.4.4 Intergeneric Hybridization

Intergeneric hybridization is generally a less preferred procedure for alien introgression, mainly due to high-level crossing barriers that prevent successful hybrid production. However, few successes have been obtained in intergeneric crosses (Table 13.4). Vassilevska-Ivanova (2005) studied wide hybridization between *Helianthus*×*Verbesina* to broaden the sunflower gene pool by introgressing new genes from diverse sources. As a result, HA-VERB, an early-flowering introgression inbred line was developed, and this line could be potentially used as a donor in sunflower breeding programs to reduce the number of days to flowering.

Christov and Vassilevska-Ivanova (1999) indicated that crosses between the line HA89 and *V. encelioides* gave hybrid seeds only when the cultivated sunflower was used as a female parent. However, crosses failed when *Verbesina* was used as a

Table 13.4 Intergeneric hybrids in sunflower

Cross combination	References
H. annuus × *Verbesina helianthoides*	Encheva et al. (2004), Seiler and Marek (2011), Christov and Vassilevska-Ivanova (1999)
H. annuus × *Verbesina encelioides* var. *exauriculata*	Vassilevska-Ivanova (2005), Christov and Vassilevska-Ivanova (1999)
H. annuus (HA89) × *Tithonia rotundifolia*	Reyes-Valdes et al. (2005), Cristov and Panayotov (1991)
H. annuus × *Echinacea purpurea*	Vassilevska-Ivanova and Naidenova (2005)

female parent. Similarly, Cristov and Panayotov (1991) used open-pollinated variety Peredovik and 3004 and HA 89 lines as female parent in *H. annuus (HA89)* × *Tithonia rotundifolia* crosses, and the intergenetic hybrids obtained were resistant to mildew, *Phomopsis*, and *Sclerotinia*, the most common diseases in cultivated sunflower. Some intergenetic hybrids between *Tithonia rotundifolia* and *H. annuus* having resistance to diseases mentioned above have also been obtained (Reyes-Valdes et al. 2005).

13.5 Successful Examples of Alien Gene Introgression

The use of wild species to improve sunflower has been successful in many cases, and consequently, genes for fertility restoration, male sterility (CMS), disease resistance (i.e., downy mildew, white rot, rusts, etc.), parasitic weed resistance (e.g., *orobanche*), stress tolerance (i.e., drought, high temperature), and new plant architecture have been introduced from wild *Helianthus* species (Korell et al. 1996; Linder et al. 1998; Iuoraş et al. 2002; Mohan and Seetharam 2005; Breton et al. 2012; Seiler et al. 2012). Genetic variation in *H. annuus* has been found to be greater than the elite germplasm, and only a small portion of this variation has been reported to be common with elite germplasm (Fick and Miller 1997). This indicates that much of the genetic improvement has been brought through wild introgression in this crop. To transfer desirable alien genes for various traits into cultivated background, numerous interspecific crosses have been made till date (Seiler 2012).

13.5.1 Yield Characters and New Plant Architecture

Variability for specific characters is necessary to make progress in breeding and selection programs aimed at improving sunflower production, especially in water-limited areas (Seiler 2008, 2011, 2012). Seed yield is directly affected by plant population, seeds per plant, and seed weight. The wild sunflower species, viz., *H. divaricatus*, *H. californicus*, *H. floridanus*, and *H. maximiliani*, have narrower and longer leaves and shorter petioles and could be used for transferring these traits in the cultivated background. Similarly, drought and stress tolerance genes could be

transferred from *H. deserticola*, *H. anomalus*, etc. (Kaya et al. 2010). Seed size and weight in sunflower are highly influenced by seed filling period, tolerance to drought and stress and then lead to higher seed weight. Similarly, the seed length should also increase (with lesser husk content), without compromising the number of seeds at head, which could be achieved by utilizing wild types and landraces. For higher oil content wild species such as *H. niveus* and *H. salicifolius* have been found to be valuable sources. Consequently, new plant types with increased leaf area led to the development of hybrids, which had greater heterosis for seed yield (Kaya et al. 2010; Seiler 2012; Škorić 2012). Some genotypes developed form interspecific crosses utilizing perennial wild sunflower species also exhibited increased number of lateral roots, mean lateral root length, and hypocotyl length.

13.5.2 Oil Content and Quality

Quality and quantity of oil are the key issues in sunflower breeding. The wild sunflower species offer considerable genetic diversity for these traits and hence are important sources for modifying oil quality traits. Scientists at the Sunflower Research Unit in Fargo, ND, USA, analyzed over 200 populations of wild sunflower represented by 5 annual and 12 perennial species, for oil content and fatty acid composition. They identified highest oil content (32.3 %) in *H. verticillatus*. They also reported linoleic acid content (83.5 %) in a population of *H. porteri*, which was also the highest ever reported for a wild annual species. Further, one population of *H. anomalous* from the desert in Utah was reported to have an oil concentration of 46.0 %, the highest reported for any wild sunflower species, followed by *H. niveus* ssp. *canescens* with 40.2 %, *H. petiolaris* with 37.7 %, and *H. deserticola* with 34.3 %. Perennial *H. salicifolius* had a concentration of 37.0 %. Knowledge of the oil and oil quality traits will facilitate the use of the wild species in breeding programs. Successful interspecific crosses were made with four annual and one perennial species (Jan et al. 2008; Seiler et al. 2010; Seiler and Jan 2010; Seiler and Marek 2011). Oleic acid, another important unsaturated fatty acid and representing high quality of sunflower oil, also appears to be quite variable in wild sunflowers. Among annual species, *H. argophyllus* had an oleic acid concentration of 475 g/kg, *H. annuus* 463 g/kg, *H. praecox* ssp. *runyonii* 410 g/kg, and *H. debilis* ssp. *cucumerifolius* 401 g/kg. Among the perennials, *H. atrorubens* had 538 g/kg oleic acid, followed by *H. hirsutus* (468 g/kg), *H. silphioides* (457 g/kg), *H. resinosus* (448 g/kg), and *H. arizonensis* (411 g/kg) (Seiler 1985, 1997, 2012). Preliminary results indicate that introgressing genes from a population of the closest wild relative can lower palmitic and stearic acid levels in sunflower oil. Reduction in the concentration of these fatty acids has been observed in a population of wild *H. annuus*, which was 50 % lower than the oil of cultivated sunflower (Seiler 2011, 2012). Similarly, a combined palmitic and stearic acid concentration of 65 g/kg was observed in a wild perennial species, *H. giganteus* (Vear 2011; Seiler 1998, 2004). Crude protein in whole seeds of perennial *H. nuttallii* ssp. *nuttallii* was observed as 348 g/kg, while protein averaged 180 g/kg in wild *H. annuus* followed by *H. porteri* as 305 g/kg (Seiler 1986).

The research for other important oil quality traits from wild *Helianthus* species led to the identification of germplasm with phytosterol content (Fernández-Cuesta et al. 2011), tocopherol profiles (Demurin et al. 1996; Velasco et al. 2004), or increased total tocopherol accumulation in the seeds (Velasco et al. 2010). Fernández-Cuesta et al. (2011) observed that wild *Helianthus* germplasm contains large variation for phytosterol content and profile that could be useful for breeding programs including these traits. The highest phytosterol content (4,308 mg/kg seed) was found in *H. praecox* ssp. *hirtus*. Several perennial species, e.g., *H. nuttallii* ssp. *nuttallii* and *H. occidentalis*, also showed higher average values of total phytosterol content as compared to the cultivated checks.

The cultivated sunflower possesses a moderate content of seed tocopherols (an antioxidant, vitamin E), predominantly made up of alpha-tocopherol. The increase of total tocopherol content in the seeds as well as the development of alternative tocopherol profiles are important breeding objectives in sunflower. Velasco et al. (2004) mentioned that the average tocopherol profile of cultivated sunflower consisted of 99.0 % alpha-tocopherol, 0.7 % beta-tocopherol, and 0.3 % gamma-tocopherol. However, wild *Helianthus* species carried significant variability for beta- and gamma-tocopherol. They also observed high level of beta-tocopherol in one accession each of *H. praecox* and *H. debilis*. Higher gamma-tocopherol level was also identified in one accession of *H. exilis* and two accessions of *H. nuttallii*. These results indicated that wild *Helianthus* germplasm contains useful variability for tocopherol profile.

13.5.3 Disease Resistance

Breeding for resistance is the most important goal in sunflower breeding as it is the best and environment-friendly mode of effective disease control. Wild sunflower species comprising both annual and perennials have been a valuable source of genes for resistance to many common pathogens (Škorić 2012; Seiler 2012). The following section discusses some of the important studies on alien gene transfer imparting disease resistance in sunflower.

13.5.3.1 Sclerotinia Stalk, Root, and Head Rot

White rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary occurs especially in humid weather conditions (Miller and Fick 1997; Seiler 2012). Many perennial species of sunflower such as *H. rigidus*, *H. mollis*, *H. resinosus*, *H. tuberosus*, *H. decapetalus*, *H. grosseserratus*, *H. nuttallii*, and *H. pauciflorus* have been shown tolerance to this disease (Rönicke et al. 2004; Block et al. 2011; Vear and Grezes-Besset 2011). However, highest resistance to *Sclerotinia* infection was observed in *H. maximiliani*. Root and mid-stalk rot tolerance was observed in perennials including *H. occidentalis* ssp. *plantagineus*, *H. pauciflorus*, *H. giganteus*, *H. maximiliani*, *H. resinosus*, and *H. tuberosus* (Škorić 1987; Kohler and Friedt 1999; Serieys et al. 2000; Seiler 2011).

At Sunflower Research Unit, Fargo, ND, USA, several crosses between cultivated lines and *Sclerotinia* stalk rot- and head rot-resistant wild perennial species have been made and also backcrossed to advance generations (Seiler 2011, 2012). For instance, hexaploid perennial *H. californicus*, which had been identified as highly resistant to *Sclerotinia* stalk rot, was crossed with the moderately tolerant public line HA 410 followed by continuous backcrossing with HA 410 and reducing to $2n = 34$ of BC progeny (Feng et al. 2006). The higher resistant stalk rot interspecific amphiploids derived from *H. divaricatus*, *H. grosseserratus*, *H. maximiliani*, *H. nuttallii*, and *H. strumosus* were crossed with HA 410 and further backcrossed twice to transfer stalk rot resistance (Feng et al. 2006; Seiler 2011). The molecular study based on SSR markers indicated a higher frequency of gene introgression from diploid perennials than from hexaploid or interspecific amphiploids, suggesting an advantage of using diploid perennials (Feng et al. 2006; Qi et al. 2011; Liu et al. 2012a).

13.5.3.2 Downy Mildew

Downy mildew (DM) caused by *Plasmopara halstedii* (Farl.) Berl. and de Toni occurs in all countries where sunflower is grown except Australia. Host-plant resistance using race-specific genes designated as *Pl*, of which 18 have been described, is the most effective way to control this disease (Seiler 2011). Resistance to this disease was reported under the control of single, race-specific major dominant genes. Multi-race-resistant germplasm and single-race-resistant germplasm from wild sunflower species have been developed (Tan et al. 1992; Miller and Fick 1997; Seiler 2011). Wild *H. annuus*, *H. petiolaris*, *H. tuberosus*, and *H. praecox* ssp. *runyonii* are the sources of dominant genes for single-race resistance, while *H. argophyllus* is the only known source of dominant genes for all current races of the fungus (Seiler 1991, 2011; Tan et al. 1992; Dussle et al. 2004; Hulke et al. 2010). *H. argophyllus*-derived germplasm carried the Pl_{arg} locus conferring resistance to all known races of DM (Seiler 1991). This gene was mapped on different linkage groups compared to all other *Pl* genes. Therefore, it could be concluded that Pl_{arg} provides a new unique source of resistance to DM (Dussle et al. 2004; Wieckhorst et al. 2010). More recently new resistant gene *Pl16* identified in HA-R4 line conferring resistance to nine races of this pathogen was mapped using SSR markers (Liu et al. 2012b). It is a potential parental source for incorporation of DM resistance into commercial sunflower lines. The closely linked markers to the *Pl13* gene in HA-R5 line provide a valuable basis for marker-assisted selection (MAS) in a sunflower breeding program. Also resistance genes to downy mildew have been reported in wild *H. petiolaris* (Christov et al. 1996) and *H. bolanderi* (Tarpomanova et al. 2009).

Alien genes for downy mildew have been introgressed from wild species in interspecific hybrids. In Bulgaria, interspecific hybrid combinations based on 9 annual species and 27 perennial species have shown resistance to downy mildew in a field screening (Christov 2008). Different progenies of interspecific hybrids with *H. pumilus* have also showed resistance to downy mildew (Nikolova et al. 2004). In sunflower, alien genes for DM were introgressed from wild *H. annuus* and *H. tuberosus*, and as

a result two cultivars, namely, Progress and Novinka, have been developed (Pustovoit et al. 1976). Interspecific hybrids with *H. salicifolius* showed complete resistance to downy mildew (Encheva et al. 2006).

13.5.3.3 Powdery Mildew

Powdery mildew, caused by *Erysiphe cichoracearum* DC. ex Meret, is a common foliar disease on senescing leaves of cultivated sunflower in warm regions of the world (Zimmer and Hoes 1978). Powdery mildew (*Erysiphe cichoracearum*) resistance was observed in annuals *H. debilis* ssp. *debilis*, *H. bolanderi*, and *H. praecox* (Saliman et al. 1982; Jan and Seiler 2007) and perennial species *H. decapetalus*, *H. laevigatus*, *H. glaucophyllus*, and *H. ciliaris* (Christov 2008). It has been reported that there are two types of resistance to this pathogen. One is controlled by a dominant gene from *H. decapetalus*, and another is controlled by multiple genes found in *H. glaucophyllus*, *H. ciliaris*, *H. laevigatus*, *H. debilis*, *H. tuberosus*, and *H. resinosus* possessing such resistance (Christov 2008). Earlier, resistance genes to powdery mildew identified in the background of *H. debilis* ssp. *debilis* (Jan and Chandler 1985) has been introgressed into a cultivated background and released a germplasm having the PM1 gene (Jan and Chandler 1988). Subsequently, complete resistance was observed in two subspecies of *H. debilis* ssp. *debilis*, *H. debilis* ssp. *vestitus*, and *H. argophyllus* (Rojas-Barros et al. 2004). These sources were completely different than those identified by Jan and Chandler (1985). Patterns of segregation for powdery mildew resistance in *H. argophyllus* and *H. debilis* confirmed the heterozygosity of the wild parent, and at least two genes have been identified to control the PM resistance (Rojas-Barros et al. 2005).

Recently reliable sources of resistance to the PM pathogen were identified in four annual wild species (*H. argophyllus*, *H. agrestis*, *H. debilis*, *H. praecox*), six perennials (*H. angustifolius*, *H. atrorubens*, *H. rigidus*, *H. salicifolius*, *H. pauciflorus*, *H. resinosus*), two interspecific derivatives (HIR-1734-2, RES-834-3), and two exotic lines (PI 642072, EC-537925) (Reddy et al. 2013).

13.5.3.4 Rust

Sunflower rust, a foliar disease caused by *Puccinia helianthi*, appears in almost all sunflower-growing regions of the world. Wild *Helianthus* species have been an important source of rust resistance genes for cultivated sunflower for several years. Resistance genes *R1* and *R2*, used widely in sunflower breeding programs, are originated from outcrossing with wild species in Texas (Seiler 2011, 2012). Recent studies indicated that two annual species, *H. annuus* and *H. petiolaris*, and five perennial species, *H. maximiliani*, *H. nuttallii*, *H. grosseserratus*, *H. pauciflorus*, and *H. tuberosus*, were free of rust (Seiler and Marek 2011). Quresh et al. (1993) reported some resistance to rust races 1, 2, 3, and 4 in *H. annuus* and *H. argophyllus*. However, in contrast to this three wild annual species, *H. annuus*,

H. petiolaris, and *H. argophyllus*, and populations derived from *H. tuberosus* were identified immune to this disease (Seiler and Marek 2011).

Qi et al. (2011) evaluated selected sunflower interspecific lines for resistance to races 336 and 777, the most virulent races currently known. One of the interspecific lines (Rf ANN-1742) was found resistant to both the races. Thus, this line has been identified as a new rust resistance source. The results indicated that the existing resistant lines are diverse in rust resistance genes, and hence durable genetic resistance, which can be developed through gene pyramiding, will be effective for the control of this disease. Recently two novel rust-resistant genes *R11* and *R12* have been identified in Rf ANN-1742 and RHA 464 lines originated from wild *H. annuus* (Qi et al. 2012; Gong et al. 2013).

13.5.3.5 Other Diseases

H. argophyllus, *H. petiolaris*, and *H. praecox* are reported to have major source for genes controlling Verticillium wilt (*Verticillium dahliae*) resistance in cultivated sunflower (Quresh et al. 1993; Seiler 2011, 2012). A line CM144, derived from an interspecific hybrid with wild *H. annuus*, has been discovered as a source of resistance to *V. dahliae* (Putt 1964). This line was subsequently used to produce released inbred lines (Fick and Zimmer 1974).

Resistance to Phoma black stem (*Phoma macdonaldii*) has been reported in several perennial species including *H. eggertii*, *H. hirsutus*, *H. decapetalus*, *H. resinosus*, and *H. tuberosus* (Encheva et al. 2012; Vear 2011). Phomopsis stem canker (*Diaporthe helianthi*) resistance has also been found in perennials such as *H. maximiliani*, *H. resinosus*, *H. mollis*, *H. pauciflorus*, *H. tuberosus*, and *H. hirsutus* and annual *H. debilis*, *H. argophyllus*, *H. niveus*, *H. neglectus*, and *H. petiolaris* (Seiler 2011). Interspecific hybrids with *H. salicifolius* have been reported to be tolerant to this disease (Encheva et al. 2006). In past years, interspecific sunflower germplasms resistant to Phoma have been developed using *H. eggertii*, *H. laevigatus*, *H. argophyllus*, and *H. debilis* (Christov 2008).

Alternaria leaf spot (*Alternaria helianthi*) resistance was observed in perennials *H. hirsutus*, *H. pauciflorus*, and *H. tuberosus* (Škorić 1985; Lipps and Herr 1986; Seiler et al. 2010; Seiler and Jan 2010). It has been reported that several wild annual species, *H. praecox*, *H. debilis* ssp. *cucumerifolius*, and *H. debilis* ssp. *silvestris*, possess high levels of resistance to Alternaria leaf spot and Septoria leaf spot caused by *Septoria helianthi* Ell. and Kell. in field evaluations (Block 1992). Madhavi et al. (2005) found *Helianthus occidentalis* and *H. tuberosus* to be highly resistant, while *H. hirsutus* was moderately resistant. Interspecific derivatives of *H. divaricatus* and cultivated sunflower have been reported to be tolerant to this disease (Prabakaran and Sujatha 2003). Using tissue culture technique, alien genes resistant to this disease have been introgressed from two hexaploid species, *H. resinosus* and *H. tuberosus*, into diploid cultivated sunflower (Sujatha and Prabakaran 2006). Wild species *H. simulans* or its derivative interspecific hybrids showed no symptoms of Alternaria leaf spot under field conditions in India (Prabakaran and Sujatha 2004).

Rhizopus head rot, caused by *Rhizopus* species, is an important disease in arid regions. It has become an important disease of sunflower in the USA (Rogers et al. 1978). The disease reduces oil quality and quantity in oilseed (Thompson and Rogers 1980). Rhizopus head rot (*Rhizopus arrhizus* Fischer) resistance was observed in perennials, viz., *H. divaricatus*, *H. hirsutus*, *H. resinosus*, and *H. laetiflorus* (Jan and Seiler 2007).

Charcoal rot, caused by *Macrophomina phaseolina* (Tassi) Goid, attacks sunflower and other crops in warm climates on all continents. Resistance to charcoal rot is positively correlated with resistance to Phomopsis stem canker and Phoma black stem (Škorić 1985). Interspecific hybrids derived using *H. tuberosus*, *H. mollis*, *H. maximiliani*, *H. resinosus*, and *H. pauciflorus phaseolina* have been found to have resistance against charcoal rot, caused by *Macrophomina* (Seiler 2011).

13.5.4 Insect Pest Resistance

Morphological, physiological, and biochemical features of host plant have been reported to be involved in the resistance to insect pests in crop plants. The antibiosis, one of the important mechanisms for insect resistance, can involve chemical resistance due to the presence of high concentrations of diterpenes, sesquiterpene lactones, coumarins, ayapin, scopoletin, and biological insecticide (*Bacillus thuringiensis*). These chemicals act as toxins and antidefendants for insects and are found abundantly in wild species of sunflower (Miller and Fick 1997; Škorić 2012). Some species also possessed inherent resistance to these pests, making it possible to search for insect resistance genes in the diverse wild species. The resistance genes have been shown mostly to control those traits, which impart resistance to insect pests. For example, seed armor layer (pericarp hardened with phytomelanin content) is an important trait that shows resistance to sunflower moth (*Homoeosoma nebulella*) (Vear 2011; Seiler 2011, 2012), and this trait has been observed in annual *H. petiolaris* and perennials such as *H. maximiliani* and *H. ciliaris*. One of the studies showed that interspecific lines, PAR 1673-1 (*H. paradoxus*) and STR 1622-1 (*H. strumosus*), had less than 2 % seed damage per head as compared to populations of *H. electellum* (Charlet et al. 2008).

Tolerance to stem weevil (*Cylindrocopturus adspersus*) was found in perennials, viz., *H. grosseserratus*, *H. hirsutus*, and *H. salicifolius*. Similarly, tolerance to sunflower beetle (*Zygogramma exclamationis*) was observed in annuals *H. agrestis* and *H. praecox* (Vear 2011). Transferring genes from these sources into cultivated background could have important and long-lasting implications in saving sunflower from damage by insects. Additionally, transgenic lines where resistance is conferred by *Bt* toxins and is specific to different insect groups have been developed in recent years (Miller and Fick 1997; Seiler 2011, 2012; Škorić 2012). Efforts are also under way to use the identified wild species to introgress resistance genes into cultivated sunflower by conventional breeding facilitated by MAS (Charlet et al. 2008; Vear 2011).

13.5.5 Broomrape Parasite Resistance

Broomrape, *Orobanche cumana* Wallr., is a parasitic weed that infects sunflower roots causing severe crop losses in Southern Europe and the Black Sea region (Kaya et al. 2012). Six resistance genes (*Or1* to *Or6*) have been deployed successfully for controlling broomrape against the A to F races. Races F has been identified recently in Spain, Turkey, Russia, Romania, and Bulgaria. It is controlled by a single dominant gene, which is capable of overcoming all earlier reported effective resistance genes (Fernández-Martínez et al. 2008; Kaya et al. 2012; Velasco et al. 2006, 2012). Velasco et al. (2006) indicated that there was the incomplete dominance of the *Or6* alleles, while segregation of germplasm derived from *H. grosseserratus* suggested the presence of a second gene, *Or7*, whose expression was influenced by environment, and based on the evaluation of $F_{2:3}$ families, digenic model of inheritance was confirmed.

Wild *Helianthus* species have been reported to possess potential genes, resistant to new virulence races of broomrape (Fernández et al. 2000, 2009; Nikolova et al. 2000; Christov et al. 2009, 2011; Hladni et al. 2011; Antonova et al. 2011; Velasco et al. 2006, 2012; Škorić 2012). Resistance to new races was found in 29 wild perennial species, while very low levels of resistance was found in annual species *H. anomalus* and *H. exilis*. Amphiploids were produced from wild perennial species *H. grosseserratus*, *H. maximiliani*, and *H. divaricatus*, and these efforts led to the release of four germplasm populations (BR1 to BR4) resistant to race F (Jan and Fernandez-Martinez 2002; Seiler and Jan 2010).

Christov et al. (2011) identified genes for broomrape resistance in 11 perennial wild sunflower species. These genes were successfully introgressed into elite cultivated lines through interspecific hybridization. Antonova et al. (2011) studied broomrape resistance of the most virulent biotypes of *O. cumana* from Rostov region of the Russian Federation and found that perennial sunflowers, viz., *H. decapetalus*, *H. laetiflorus*, *H. californicus*, *H. giganteus*, *H. grosseserratus*, *H. salicifolius*, and *H. occidentalis*, are immune to broomrape. Velasco et al. (2012) observed that an accession of *Helianthus debilis* ssp. *tardiflorus* exhibited complete resistance to race G controlled by dominant alleles at a single locus. Monogenic, dominant inheritance is particularly useful in the development of hybrid varieties (Fernandez et al. 2009). Transfer of resistance to broomrape in cultivated sunflower from wild species with different ploidy levels is practically possible both in the F_1 and backcross generations (Sukno et al. 1998).

13.5.6 Herbicide Tolerance

Herbicide-resistant crop varieties are becoming increasingly popular in agricultural production worldwide. Resistance to herbicides such as *imazethapyr* and *imazamox* holds great promise for producers in all parts of the world for controlling several

broad leaf weeds (Kaya et al. 2012). A population of wild *H. annuus* from a soybean field in Kansas that had been repeatedly treated with imazethapyr for 7 consecutive years developed resistance to the *imidazolinone* (IMI) and *sulfonylurea* (SU) herbicides (Al-Khatib et al. 1998), which may also control broomrape parasitic weed in infested areas. The USDA-ARS research group quickly transferred this genetic resistance into cultivated sunflowers by backcrossing and developed and released the populations of IMISUN-1 (oil maintainer), IMISUN-2 (oil restorer), IMISUN-3 (confection maintainer), and IMISUN-4 (confection restorer) (Al-Khatib and Miller 2000; Seiler and Marek 2011). One oilseed maintainer and two fertility restorer-breeding lines with IMI herbicide resistance have also been released (Miller and Al-Khatib 2002). Genetic stocks SURES-1 and SURES-2 with resistance to the SU herbicide tribenuron have been developed and released (Miller and Al-Khatib 2004). The commercial hybrids having these new alien genes derived from wild species have sustained successfully in sunflower production for the last 8 years (Kaya et al. 2012). On the other hand, the wild sunflower populations (*H. annuus* and *H. petiolaris*) as well as some populations from the USA and Canada have been screened for resistance to these herbicides, and some resistance to imazamox and tribenuron have been determined (Seiler and Marek 2011).

13.5.7 Abiotic Stresses: Drought and Salt Tolerance

Sunflower is generally grown in marginal soils, often in semiarid conditions, where abiotic stresses always act as a major limiting factor for its production and productivity in many parts of the world (Škorić 1988). Therefore breeding for resistance to drought and high temperature is an important goal in most of the sunflower breeding programs (Fick and Miller 1997; Škorić 2012). It has been shown that wild *Helianthus* species, especially *H. argophyllus* and *H. paradoxus*, have genetic diversity for these stresses and hence offer great possibilities for increasing the genetic resistance of the cultivated sunflower towards abiotic stresses and salinity (Škorić 2009). Other wild species such as *H. deserticola*, *H. hirsutus*, *H. maximiliani*, and *H. tuberosus* could also be used for drought resistance. The germplasm derived from *H. argophyllus* through several cycles of intercrossing showed great value for sunflower breeding due to their resistance to drought with higher yield and stability (Griveau et al. 1996). In perennial wild species, higher diffusive resistance, transpiration rate, and stomatal densities have been observed compared to the annual species (Seiler 1992; Seiler et al. 2008, 2010). The pubescent leaves of *H. argophyllus* reflect sunlight, reduce water loss, and exhibit low transpiration rates for increased and reduced transpiration, and hence it can be a potential source of drought tolerance in cultivated sunflower (Škorić et al. 1995; Škorić 2009).

Several species of *Helianthus* are native to salt-affected habitats and therefore may possess genes for salt tolerance. Seiler et al. (1981) suggested that *H. paradoxus* growing in saline marshes in the USA would be a likely candidate for salt tolerance genes. Chandler and Jan (1984) evaluated three wild *Helianthus* species, viz., *H. paradoxus*, *H. debilis*, and wild *H. annuus* populations which are native to salty

desert areas for salt tolerance. *H. paradoxus* had the highest salt tolerance ability, with some plants surviving at 1,300 mM of salt concentration. The hybrids between *H. paradoxus* and cultivated sunflower were found to be five times more salt tolerant than the parents (Škorić 2009). More recently, salt tolerance genes have been identified and transferred into cultivated sunflower, and two salt-tolerant parental oilseed maintainer lines, HA 429 and HA 430, have been released (Jan and Seiler 2007). Study showed that one major dominant gene controls salt tolerance, although a recessive modifier gene may also be present (Miller and Fick 1997).

13.5.8 Male Sterility and Fertility Restoration

Two types of male sterility systems, viz., nuclear male sterility (NMS) and CMS, occur in sunflower. NMS is generally a result of a single recessive gene pair, and its source was identified in wild species, *H. grosseserratus* (Jan and Seiler 2007). However, this male sterility system is not used commonly in hybrid production in sunflower except for some early testing (Fick and Miller 1997). Currently, all sunflower hybrids are CMS based in the world, which is derived from single source of wild species *H. petiolaris* (PET1 by Leclerq and Whelan's CMS cytoplasm from *H. petiolaris*, PET2; however, due to enhancement in the genetic vulnerability of sunflower hybrids to various diseases and insect pests, efforts are being made to find new CMS sources and fertility restorer genes from the wild species) (Christov 2012). Consequently, CMS sources have also been obtained by intraspecific and interspecific hybridizations (wild × cultivated species) (Škorić 2012; Christov 2012). Their fertility restoration was found to be 100 %, and it was controlled by a single gene. Two fertility restoration genes (*Rf1* and *Rf2*) for this source of CMS have been used exclusively for sunflower hybrid production worldwide (Fick and Miller 1997). Similarly, the *ARG1* and *ARG3* derived from one wild accession of *H. argophyllus* were also fully male sterile without any negative effects and had similar restoration pattern as that of CMS-PET1 (Christov 1991; Jan and Seiler 2007). Serieys (1994) also reported complete male sterility and full fertility restoration by single dominant genes for CMS-ANO1, CMS-NEG1, and CMS-PRP1. However, the utilization of these CMS sources for potential hybrid production is yet to be pursued on a commercial scale.

To date, 70 CMS sources have been identified from progenies of crosses made between wild *Helianthus* accessions and cultivated lines or from induced mutations. Fertility restoration genes have been reported for 34 CMS sources, while detailed inheritance studies have been conducted for only 19 CMS sources (Jan and Seiler 2007). Most of these genes are from wild relatives and coded with three letter each, viz., *H. petiolaris* ssp. *fallax* (PEF1), *H. resinosus* (RES), *H. rigidus* (RIG), *H. giganteus* (GIG1), and *H. maximiliani* (MAX1) (Serieys and Christov 2005), CMS ARG-3, etc. (Christov 1991). Serieys and Christov (2005) indicated that CMS-PET1, ANL1, ANN1, ANN2, ANN3, ANN4, BOL1, and MAX1 had complete male-sterile heads with degenerated anthers over the locations.

Some other CMS fertility restoration systems have also been identified in which the fertility restoration was controlled by more than one gene (Serieys and Christov 2005;

Whelan 1980; Serieys and Vincourt 1987; Christov 1991, 2008), while some others were reported from experimental mutagenesis (Christov 1993, 1994, 1999) and spontaneous occurrence of sterile plants (Christov 1993, 1999; Jan 1997). Qi et al. (2012) mentioned that *Msc1* and *Rf3* reported recently in RHA 340 and RHA 280 lines are also able to restore CMS PET1 (Jan and Vick 2007; Liu and Jan 2012), whereas the *Rf4* gene is specific for male fertility restoration in CMS GIG2, a system different from CMS PET1 (Feng and Jan 2008). Continuous backcrossing of these male-fertile segregants with non-restoring cultivated lines will lead to the production of male-fertile and male-sterile isogenic lines differing by the restoration genes. To simplify the comparison between the male-sterile and male-fertile cytoplasm, it would be much easier to compare isogenic lines of the cytoplasmic genome instead of isonuclear lines as presently used.

13.6 Negative Impact of Alien Gene Introgression

Intercrossing between cultivated and weedy sunflower rises as a risk in recent years both for the evolution of more aggressive weeds especially in North America and also for maintaining wild *Helianthus* collections around the world. These wild *H. annuus* weeds probably came from pollution of basic and hybrid seed production in North America which affected around 15 % of sunflower fields in the area studied and caused 50 % yield losses. Weedy forms morphologically close to wild *H. annuus* have been observed in France and other European countries in recent years (Vear 2011; Burke et al. 2002; Seiler 2011, 2012).

13.7 Future Perspectives of Alien Gene Introgression

Traditional breeding has resulted in the development of high-yielding hybrids that have adaptability to marginal areas. At the same time, novel molecular breeding tools and methods have opened new opportunities for traditional plant improvement. Advances in sunflower genomics and molecular marker technology have accelerated alien gene introgression in sunflower (Durante et al. 2002; Sarrafi and Gentzbittel 2005; Pérez-Vich and Berry 2010; Hahn and Wieckhorst 2010; Kiani and Sarrafi 2010; Anisimova et al. 2009, 2011; Dyrszka et al. 2012). These advances have made it possible to assess the agronomically important alien genes, which could otherwise not be possible through traditional breeding methods.

13.7.1 Molecular Marker Technology

Advanced backcross-QTL strategy is one of the important strategies used widely for introgression of alien genes from wild species (Al-Chaarani et al. 2004; Pérez-Vich

and Berry 2010; Hu 2010; Liu and Jan 2012). This method quickly discovers and transfers useful QTLs from non-adapted to adapted germplasm (Vischi et al. 2001). Favorable QTL alleles originating from four different wild relatives have been identified for important agronomic traits (Liu and Jan 2012). Gandhi et al. (2005) mapped three seed dormancy QTLs to linkage groups in a wild × domesticated sunflower backcross population, but only sampled one post-harvest storage stage and 12.1 % of the phenotypic. AB-QTL analysis resulted in identification of genetic factors that limited pollen viability in interspecific hybrids (backcross of the F_1 interspecific hybrid *H. annuus* × *H. argophyllus*) (Quillet et al. 1995). Lexer et al. (2003) reported a QTL locus analysis of mineral ion uptake traits and survival in BC_2 hybrids between *H. annuus* and *H. petiolaris* planted in *H. paradoxus* salt marsh habitat in the USA. Pizarro et al. (2006) developed QTL-NILs varying for target QTLs for seed oil concentration by backcrossing a donor parent (a wild *H. annuus*) to a recurrent parent (elite high oil cultivar) combined with MAS. Comparison of phenotypes of the QTL-NILs with the recurrent parents allowed to obtain an accurate evaluation of the effects of the target QTL in an adapted background. Lexer et al. (2005) analyzed an extensive QTL dataset for an interspecific backcross between two wild annual sunflowers, *H. annuus* and *H. petiolaris*, and they indicated that morphological traits, particularly flower morphology, were strongly and consistently selected than physiological traits.

Molecular mapping of both rust resistance and male fertility restorer genes in the background of wild species has been conducted to accelerate the introgression of these genes into elite cultivars and pryramiding of R-gene in sunflower. Qi et al. (2012) mapped a novel rust resistance gene, *R11*, and a male restorer gene, *Rf5*, tightly linked in the coupling phase in the background of wild *H. annuus*. Similarly, Gong et al. (2013) also discovered an *R12* novel rust resistance locus in sunflower RHA 464 restorer line originated from wild *H. annuus* and developed associated SSR markers with this gene. The molecular markers also helped to identify the alien segments in the background of cultivated lines. For example, progenies of partial hybrids between *H. maximiliani* resistant to *S. sclerotorium* and *H. annuus* were characterized on the basis of AFLP markers in order to determine the introgressions from *H. maximiliani* into cultivated sunflower at molecular level. Some of the progenies showed a higher level of resistance in comparison with the resistant inbred lines. Thus molecular markers associated with these genes are potentially supporting the molecular marker-assisted introgression and pyramiding of alien gene into sunflower breeding.

13.7.2 Protoplast Technology

Sunflower protoplasts have been isolated from various tissues, for example, root (Bohorova et al. 1986; Henn et al. 1998; Binsfeld et al. 2000; Rákosy-Tican et al. 2007) and hypocotyl or cotyledon and mesophyll tissues (Krasnyanski and Menczel 1995; Taski-Ajdukovic et al. 2009; Horn and Hamrit 2010; Kativat et al. 2012), for generation of somaclonal variants to form interspecific or intergeneric hybrids.

The application of protoplast fusion has been reported previously for the transfer of *Sclerotinia sclerotiorum* resistance genes from wild *Helianthus* species to cultivated sunflowers to produce resistance hybrids (Krasnyanski and Menczel 1995; Henn et al. 1998; Taski-Ajdukovic et al. 2006). Nevertheless, successful development of protoplast-to-plant systems is still limited because it usually depends on various factors, especially genotypes (Krasnyanski and Menczel 1995; Horn and Hamrit 2010; Kativat et al. 2012). The optimization of various factors has led to improved protoplast yields. Kativat et al. (2012) developed the most responsive protoplast isolation procedure. Similarly, the same authors also obtained the highest protoplast yield from hypocotyls as compared to the previous reports (Krasnyanski and Menczel 1995; Taski-Ajdukovic et al. 2006).

13.7.3 Doubled Haploids

Several studies have been conducted on the use of in vitro techniques for production of haploid and doubled-haploid (DH) plants in cultivated sunflower through anther culture (Gürel et al. 1990; Vasić et al. 2000; Nenova et al. 2000), pollen culture (Gürel et al. 1991; Todorova et al. 1993), and ovule culture (Horn and Hamrit 2010). However, none of these techniques could be successfully applied to routine breeding programs due to low regeneration ability (Ivanov et al. 2002; Durante et al. 2002). Todorova et al. (1997) were successful in producing double haploids in sunflower based on γ-induced parthenogenesis.

A set of genetically diverse cultivated lines, wild *Helianthus* accessions, amphiploid hybrids, and interspecific amphiploids such as *H. maximiliani*, *H. pumilus*, and *H. hirsutus* have been subjected to anther and microspore culture. Distant crosses were also attempted between CMS and several cultivated lines, and the CMS F_1 plants were pollinated by pollen of *H. tuberosus* accessions to observe any embryo formation and haploid induction through preferential chromosome elimination (Jan et al. 2011).

13.7.4 Genetic Transformation

Genetic transformation in sunflower is difficult due to low regeneration rate and high genotype dependency. Most investigations on genetic transformation of sunflower have used the neomycin transferase (nptII) gene as the selectable marker (Cantamutto and Poverene 2010), and shoot apical meristems have been mostly used as the explants (Burrus et al. 1996; Lucas et al. 2000; Neskorodov et al. 2007; Horn and Hamrit 2010). Improved transformation protocols using incubation of apically expressing *Agrobacterium* EPSPS gene *cp4* and *B. thuringiensis* enthomotoxin gene *cry1A* for control of Lepidoptera (known Bt) and oxalate–oxidase gene for control of *Sclerotinia* (known as oxox) have been developed. The transgenics developed through these techniques were subjected to the field trials in the USA and Argentina. However, they have not been put to commercial production yet (Paniego et al. 2007).

13.8 Conclusion

Sunflower production continues to be pushed into low-fertility soils and other marginal environments where biotic and abiotic stresses continuously affect sunflower production reducing its yield. The challenge for future breeding programs is to develop high-seed- and -oil-yielding cultivars adaptable to these marginal environments (Seiler 2011; Škorić 2012). Wild species of sunflower are a rich source of useful genes to increase genetic variability. Significant advances have been made in understanding the origin, domestication, and organization of the genetic diversity as well as characterization and screening for abiotic and biotic stresses in sunflower (Seiler 2012). The studies have clearly demonstrated that large genetic diversity exists in *Helianthus* genus, and an amalgam of the conventional breeding methodologies and modern techniques is required for successful exploitation of this variability for the genetic improvement of cultivated species (Liu and Jan 2012). The future efforts need to include the transfer of target genes from wild relatives into cultivated ones with improved genetic backgrounds adapted to local conditions by introgression of favorable alleles from alien germplasm, pyramiding favorable alleles and deployment of QTLs for specific traits. Simultaneously application of molecular marker technology needs to be put to its best for maximization of seed and oil yield and oil quality. Our ability in enriching the allelic diversity of elite breeding lines is expected to become one of the key factors for competitive sunflower breeding programs in the future. Phenotypic screening in various environments is needed to be followed by precise genotyping for target traits and QTL discovery and mining for generating elite QTL-inbreds, which in turn can be used directly, for producing both new commercial varieties as well as sources of exotic QTLs for using in other breeding materials (Dyrszka et al. 2012; Liu and Jan 2012).

Conventional breeding and selection for oil-related traits in sunflower and use of a single CMS source for commercial production of hybrids have resulted in drastic reduction in genetic diversity of this important oil crop. Therefore, future efforts will require the exploitation of wild germplasm as a source of novel alleles (Chapman and Burke 2012). In order to keep sunflower an economically viable oil crop globally, researchers must attempt to combine the best conventional and novel molecular methods with multidisciplinary team approach and a commitment to a long-term integrated genetic improvement program utilizing alien gene introgression.

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Chapter 14
Sugarcane

Phillip Jackson, Anna Hale, Graham Bonnett, and Prakash Lakshmanan

Abstract Sugarcane is one of the most important crops globally, providing most of the world's sugar and bioenergy (ethanol and electricity). This contribution has been underpinned by the successful introgression of genes from wild germplasm, particularly from *Saccharum spontaneum*, by breeders in the early 1900s. This introgression resulted in a step change in the vigour, ratoon growth (i.e. regrowth after harvest), and adaptation to adverse environments, compared with the existing *S. officinarum* varieties. Introgression of other *S. spontaneum* clones and other species (particularly *Erianthus* spp. and *Miscanthus*) related to sugarcane in a range of sugarcane breeding programmes around the world is continuing, and based on current reports it is expected to continue to contribute incrementally to gains in breeding programmes and cultivar performance. However, the low sugar content of most wild relatives means that several cycles of backcrossing (to commercial type sugarcane) and interim selection are required, and this makes investment in these programmes lengthy, costly, difficult, and risky. Technological advancements in GM research have been impressive in sugarcane with a variety of methods to introduce and express genes in sugarcane now available. So far no commercially successful outcomes of this technology have occurred, but some major programmes are currently underway aiming to develop commercial cultivars. Targets include herbicide tolerance, stem borer resistance, and production of foreign compounds (e.g. alternative sugars).

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14.1 Introduction

Sugarcane is a crop of major economic importance produced in many countries in the tropics and subtropics and is harvested in greater quantities than any other crop globally (http://faostat.fao.org). Over 100 countries grow sugarcane commercially, the top three being Brazil, India, and China. Over 1.5 billion tonnes of sugarcane is harvested and processed annually. World sugarcane production has approximately doubled over the last 25 years largely due to the expansion of sugarcane cultivation in Brazil. This global expansion has been driven by both a long-term trend increase in sugar consumption of around 2 % per annum and the use of sugarcane for ethanol production in Brazil.

A major objective of sugarcane improvement programmes has been and remains to increase levels of commercially extractable sucrose content in cane. In the production of raw sugar, high levels of fibre and soluble impurities in cane cause losses through increased juice extraction costs and loss of sucrose in processing. Because of this, the first commercially produced sugarcane varieties of the species *Saccharum officinarum* had high sucrose content and low fibre and impurity levels. As described below, a major progression in sugarcane improvement arose with introgression of components of the *S. spontaneum* genome and other species into *S. officinarum* cultivars. *S. spontaneum* provided sources of disease resistance, vigour, ratooning ability, and better yields under abiotic stresses (Fig. 14.1).

Current highest priority objectives of most modern sugarcane breeding programmes include maintaining or improving disease and pest resistance and improving commercially extractable sugar content, cane yield, and ratooning performance. Most breeding programmes focus on parental material based on known original clones and well-defined (average of eight to nine) prior cycles of breeding and selection. Most parental materials in sugarcane breeding programmes around the world are derived from a limited number of ancestors produced during the initial interspecific hybridisation of the early 1900s described above. Some concerns about the narrow sampling of ancestral clones in modern sugarcane breeding programmes have been expressed by sugarcane breeders (Roach 1989). This prompted periodic attempts by breeders at introgression of related species as described below, with mixed success. More recently researchers in some institutes, as in many crop species, have turned to introduce alien genes into sugarcane cultivars through genetic engineering approaches, particularly for sucrose accumulation, pest resistance, and herbicide resistance. The aim of this chapter is to provide an overview of past and current efforts to introgress alien genes into sugarcane improvement programmes, either using sexual hybridisation with wild species or through genetic engineering approaches. A brief overview of sugarcane genetics and breeding and the early

Fig. 14.1 Modern sugarcane (*top*) cultivars are derived from *Saccharum officinarum* (*below left*) and the wild species *S. spontaneum* (*below right*)

introgression of wild canes into the original sugarcane cultivars are given in Sects. 14.2 and 14.3.1. Following this, more recent efforts and research aimed at further introgression of favourable alien genes from related species are described in Sects. 14.3–14.5. In Sect. 14.6, an overview of genetic engineering approaches taken to date in sugarcane improvement programmes is described. Lastly in Sect. 14.7 some perspectives on potential future approaches are provided.

14.2 Biology and Breeding

14.2.1 Taxonomy

The taxonomy of sugarcane and its relatives remains complex and controversial (Kellogg 2012). Sugarcane cultivars belong to the genus *Saccharum*, within the tribe *Andropogoneae*. This tribe is frequently polyploid, and its speciation and evolution are unclear in many cases, with the monophyletic status of many genera being questioned in some studies (Hodkinson et al. 2002). Further it is difficult to define taxonomic boundaries because of frequent interspecific and inter-generic hybridisation. Mukherjee (1957) first used the term "*Saccharum* complex" to describe an interbreeding group implicated in the origin of sugarcane and was adopted by other sugarcane breeders and geneticists, including Daniels and Roach (1987) who provided a comprehensive review of the members of this complex. Apart from *Saccharum*, the related genera include *Erianthus*, *Miscanthus*, *Narenga*, and *Sclerostachya*.

Traditionally six species were recognised within the genus *Saccharum* (Naidu and Sreenivasan 1987), with two of these growing in the wild (*S. spontaneum* and *S. robustum*) and the other four (*S. officinarum*, *S. barberi*, *S. sinense*, *S. edule*) existing primarily in cultivation.

Earliest sugarcane industries in the world grew varieties of *S. officinarum*. This species is typically characterised by high sugar content, low fibre content, thick stalks, and broad leaves. It originated in New Guinea and/or nearby Melanesian or Polynesian islands (Mukherjee 1957; Brandes 1958) and is believed to have evolved from *S. robustum* (Grivet et al. 2006).

Modern cultivated sugarcane varieties are complex interspecific hybrids primarily involving *S. officinarum* and *S. spontaneum* and in most cases involving some contributions from *S. robustum*, *S. sinense*, *S. barberi*, and possibly other related genera (Daniels and Roach 1987). *S. barberi* and *S. sinense* comprise the ancient landraces of India and China, respectively, and could have developed from interspecific hybrids involving *S. officinarum* and *S. spontaneum* (D'Hont et al. 2002) with possible introgression from other genera (Brown et al. 2007).

S. spontaneum ($2n = 36–128$) is distributed widely in the tropics to the subtropics in Asia and Africa in a wide diversity of habitats. It is a highly variable species that is found in the wild and varies in appearance from short bushy types to large

stemmed clones over 5 m in height. Most *S. spontaneum* clones have thin stalks and leaves, low sugar content, and high fibre content.

The other wild species in the genus, *S. robustum*, has some characteristics similar to *S. officinarum* but with generally lower sugar content and more variability (Daniels and Roach 1987). *S. edule* is a species characterised by an aborted inflorescence and therefore cannot be used for breeding.

Irvine (1999) challenged this traditional division of *Saccharum* into six species arguing that there is little basis for the separation and that the six species should more properly consist of two: one being *S. spontaneum* and the other comprising the five other species to be called *S. officinarum* (Irvine 1999).

14.2.2 Genetics

The genome of modern sugarcane is widely recognised as being the most complex of all important crop species (Grivet and Arruda 2001). A major feature of the sugarcane genome is its high degree of ploidy and aneuploidy and two (although not entirely separate—see below) subsets of chromosomes arising from the two main progenitor species, *S. officinarum* and *S. spontaneum*. *S. officinarum*, the dominant progenitor of sugarcane, is an octoploid with a basic chromosome number of $x=10$ (D'Hont et al. 1996, 1998). The other main contributor to the sugarcane genome, *S. spontaneum*, has a basic chromosome number of $x=8$ (D'Hont et al. 1996, 1998) and a range of cytotypes from $2n=36$ to $2n=128$, with five major cytotypes of $2n=64$, 80, 96, 112, and 128 (Panje and Babu 1960). Modern sugarcane cultivars which are derivatives of interspecific hybridisation involving these two species are complex aneu-polyploids, comprising 70–80 % of *S. officinarum*, 10–20 % of *S. spontaneum*, and 5–20 % of recombinant chromosomes (D'Hont et al. 1996; Piperidis and D'Hont 2001).

An interesting feature of crosses between *S. officinarum* and *S. spontaneum* is the transmission of $2n$ chromosomes from *S. officinarum* (Bremer 1923), which contributes to the high ploidy of modern cultivars. From a review of literature and breeder's experience, this phenomena seems to occur when *S. officinarum* as a female parent is crossed with any clone containing some *S. spontaneum* chromosomes.

The high level of polyploidy of sugarcane contributes to a very large genome size: the non-replicated size of *S. officinarum* is estimated at 7,440 Mb (Grivet and Arruda 2001), while the monoploid genome size of 930 Mbp is comparable with a range of diploid crops.

Genetic linkage maps are difficult to construct because of the high level of polyploidy. The usage of single-dose markers (i.e. markers present in a single copy and therefore that segregate 1:1 in gametes) provided an avenue for construction of linkage maps based on this subset of markers (Wu et al. 1992). Current sugarcane maps containing in excess of 1,000 linked markers are available (Hoarau et al. 2001; Aitken et al. 2005) but are still incomplete and unsaturated. Presence of multiple copies of some linkage groups may make completion using genetic mapping

difficult because of the dependence of low copy number markers (single- or double-dose markers) using current approaches. The meiosis of sugarcane cultivars mainly involves bivalent pairing (Price 1963). Genetic linkage maps of *S. officinarum* and modern cultivars have indicated random pairing of chromosomes combined with some preferential pairing (e.g. Aitken et al. 2005, 2007a, b; Hoarau et al. 2001).

A range of QTL mapping studies have been performed in sugarcane (listed and reviewed by Pastina et al. 2010). Analyses on a range of traits, including brix, sucrose content, fibre, cane yield, and disease resistance have been conducted. The general result reported is of multiple small QTL explaining proportions of variation (r^2) from 2 to 22 %. However, the values in the higher ranges are in studies with relatively small numbers of genotypes and are most likely overestimates, considering the methods used. Only a small number of traits having a major effect controlled by a single gene have been reported in sugarcane, with examples provided by D'Hont et al. (2010) and Costet et al. (2012).

14.2.3 Brief History of Breeding

The development of plantation and factory-based sugarcane industries in the 1800s and up to the early 1900s were based on clones of *S. officinarum*, mainly because of the superior processing attributes of these clones. This included low fibre content and low levels of impurities in juice (non-sucrose-dissolved solids). The fertility of sugarcane was discovered in the late 1800s, and following this, breeding programmes were quickly developed. Historically, three main phases have been identified in professionally directed sugarcane breeding programmes conducted since the late nineteenth century (Roach 1989). The first was intraspecific hybridisation of selected *S. officinarum* clones. Generally the goal was more disease-resistant forms of *S. officinarum* with good factory qualities and improved yield. Apart from the favourable processing attributes of *S. officinarum*, clones from this species are also usually characterised by poor vigour under abiotic stresses, poor ratooning ability, and susceptibility to a range of diseases.

The second phase which occurred between 1912 and around the 1930s was interspecific hybridisation involving mainly *S. officinarum* × *S. spontaneum* clones and is described in more detail in Sect. 14.3.1. It was also found that resulting hybrids were generally more vigorous, were more tolerant to a range of environmental stresses, and had stronger ratooning, compared with the prior *S. officinarum* cultivars. Following initial interspecific hybridisation, breeders found it necessary to backcross the hybrids to *S. officinarum* to "dilute" the undesirable characters in the wild canes, particularly low sugar content. This backcrossing process is termed "nobilisation" by sugarcane breeders.

The third phase, beginning around the 1930–1940s and continuing until today, involved exploiting the material produced in the second phase. This has involved intercrossing among the original hybrids and recurrent crossing and selection among progeny with increasingly larger populations. Most crosses made in

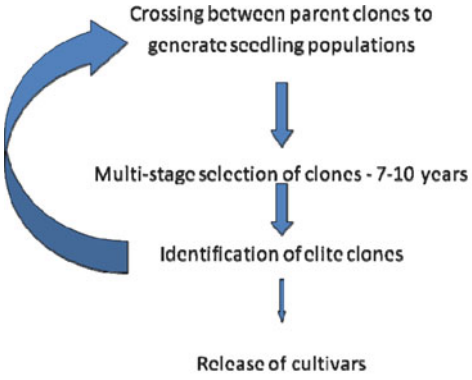

Fig. 14.2 General scheme of operations in most commercial sugarcane breeding programmes

breeding programmes throughout the world today are based on a relatively small number of ancestors derived from the early interspecific hybrids produced in the second phase.

14.2.4 Objectives and Structure of Breeding Programmes

There are currently around 40 significant sugarcane breeding programmes around the world, mostly specifically targeting sugarcane industries within individual countries (Machado 2001). The three traits of highest overall importance are resistance to prevailing diseases and pests, high commercially extractable sucrose content, and high cane yield in plant and ratoon crops. Some programmes also pursue traits associated with ease of harvesting and crop management (e.g. fast canopy cover to control weeds). A detailed account of many important aspects of sugarcane breeding, including important practical aspects, was provided by Heinz (1987). Most programmes follow the general scheme outlined in Fig. 14.2.

A heavy weighting in selection has been applied to commercially extractable sugar content in sugarcane breeding programmes and is justified for two reasons. First, any given incremental contribution of higher sugar content on sugar yields is economically more valuable than the same contribution by cane yield because increases in cane yield also give rise to higher harvesting, transport, and milling costs, in contrast to minimal marginal costs associated with increased sugar content (Jackson et al. 2000). Second, sugar content usually has a higher degree of genetic determination than cane yield (Skinner et al. 1987; Jackson and McRae 2001).

High fibre content in cane impacts adversely on sugar production systems through increased loss of sucrose in bagasse and on milling rate by slowing it down. However, in future production systems producing energy (electricity or ethanol) from fibre, production of additional fibre above that needed for factory processing energy requirements may have a positive value. This may have important implications for sugarcane introgression breeding programmes in that early generation progeny

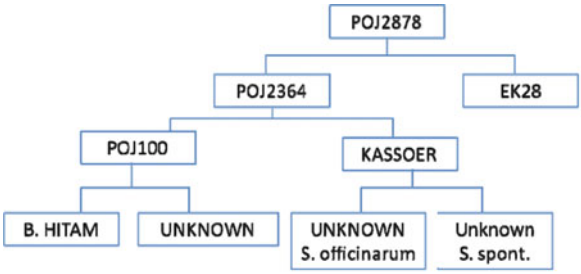

Fig. 14.3 Pedigree of clone "POJ2878" (from Mangelsdorf 1960) "*B Hitam*" (full name *Bandjermasin Hitam*) is a *S. officinarum* clone, which was open pollinated to produce the cultivar POJ100. POJ100 was crossed with the naturally occurring hybrid between *S. officinarum* and *S. spontaneum*, Kassoer, to produce POJ2363 which was crossed with the *S. officinarum* cultivar EK28

will have higher relative economic value than in production systems targeting only sucrose production, and therefore introgression of new traits from wild canes may be easier than in the past.

14.3 Introgression from *S. spontaneum*

14.3.1 Early Introgression

The introgression of genome components from *S. barberi* and *S. spontaneum* into *S. officinarum* in sugarcane breeding programmes in the early 1900s represents one of the most important and successful examples of introgression of wild relatives in any major crop species. Introgression of *S. spontaneum* into *S. officinarum* using deliberate hybridisation and selection within sugarcane breeding programmes occurred in both Indonesia and India.

In Indonesia, obtaining resistance to the diseases "serah" and sugarcane mosaic virus from *S. spontaneum*-related clones was an important motivating factor contributing to the early interspecific breeding efforts, and this was successful. However it was found that the interspecific hybrids and derivatives were also more vigorous and adaptable to environmental stresses and had better ratooning ability compared with the best *S. officinarum* clones.

The most famous clone produced from the early introgression programme in Java was POJ2878 (Fig. 14.3), also known as the "Java wonder cane". Development of this clone is described by Mangelsdorf (1960). In 1893 the breeder J.H. Wakker grew seed from open pollination of the typical *S. officinarum* clone "Bandjermasin Hitam", and from this population, he selected the variety POJ100 which became a major commercial variety in Java. In 1911, a sugarcane pathologist, Miss G. Wilbrink, became interested in the clone Kassoer as a source of resistance for serah disease which was important in the Java sugar industry up to that time. Kassoer was

later shown to be a natural hybrid between *S. officinarum* and *S. spontaneum*. Wilbrink crossed POJ100 with Kassoer and amongst this population a clone 'POJ2364' was selected. This clone although was immune to Serah disease, but had too low in sugar content for commercial production. In 1917 the breeder J. Jesweit crossed POJ2364 with the important *S. officinarum* cultivar EK28 and obtained several promising clones. In subsequent years he generated more clones from this cross, and from a cross made in 1921 he selected POJ2878. The superiority and reported rate of extension of this variety seem to be astonishing; within 8 years this variety occupied over 400,000 acres or 90 % of the production area in Indonesia, with reportedly a 35 % yield advantage over the varieties it replaced (Mangelsdorf 1960). This clone is exceptional also in its use in subsequent breeding efforts and is in the ancestry of most commercial cultivars around the world today.

In 1919, Dr. Elmer Brandes of the Office of Sugar Plant Investigations, Bureau of Plant Industry, USA, identified sugarcane mosaic virus in Louisiana (USA) and began to identify varieties resistant to the disease. Resistant POJ varieties were imported to the Southdown plantation, near Houma, Louisiana, through Washington, D.C. in 1922 and 1923. Local lore described the varieties as the "Please Oh Jesus" (POJ) varieties, because they were seen as one of the last hopes for a nearly bankrupt industry with heavy mosaic pressure. By 1924, the varieties were recognised as the salvation of the local industry, and the newly imported varieties (POJ234, 36, and 213) were described as "… a prosperous but self-centered oasis in a mosaic desert …" (Abbott 1971). Many other sugarcane industries around the world experienced similar results with the introduction of the POJ varieties, and local breeding programmes started using these varieties as parental material.

In India in 1912 the first recorded deliberate cross between *S. officinarum* and *S. spontaneum* was made at the Sugarcane Breeding Institute, Coimbatore, under the leadership of Dr. C.A. Barber and Sir T.S. Venkataraman (Nair 2008). This cross between Vellai (*S. officinarum*) and a wild *S. spontaneum* produced the interspecific hybrid Co205, which was released as a commercial cultivar in the Punjab province of northern India in 1918. This clone reportedly yielded 50 % more than the *S. barberi* cultivars it replaced in that region (Thuljaram Rao 1987, cited in Selvi et al. 2005). This clone was clearly better adapted to the challenging climatic conditions in the subtropical region it was released to. Other interspecific hybrids were also produced in India at around the same time and were crossed with *S. officinarum* clones and other hybrids between *S. officinarum* and either *S. spontaneum* or *S. barberi*, including POJ clones introduced from Java. These led to other important cultivars suited to production environments both in India and worldwide such as Co281 and Co290. These in turn led to further significant cultivars and parents such as Co331, Co419, Co421, and Co475, many of which also feature in ancestries of many important sugarcane cultivars worldwide today.

Between 1919 and 1939, the idea of using wild species in breeding programmes became more defined, and a serious effort was made to assemble a collection of sugarcane from around the world, both from breeding institutions and from indigenous regions (Brandes et al. 1939). Stated eloquently by Brandes (1935), "The pursuit of knowledge and the hope that such researches may eventually lead to production of

crop plans of economic importance is the double stimulus which prompts the attempts to secure and study these hybrids. The expenditure of effort and money in crossing the large thick-stemmed, tropical sugarcanes with the slender, unprepossessing wild cane *Saccharum spontaneum* has already paid enormous dividends". The development of commercial sugarcane is an example of the usefulness of germplasm enhancement programmes and gains that can be made through breeding with related wild species.

14.3.2 Later (Post 1960) Introgression of S. spontaneum

Following the successes and rapid genetic gains in cane yield, ratooning performance, and adaptation to marginal (especially cool) environments from the initial introgression of *S. spontaneum* in sugarcane breeding programmes, sugarcane breeders continued to exploit the early interspecific clones developed and their selected progeny. This involved intercrossing among the original hybrids and recurrent crossing and selection among progeny with increasingly larger populations, following the general process outlined in Fig. 14.1.

Following the generation of these original hybrids, few efforts were made in several subsequent decades to broaden the genetic base of sugarcane. However, around the 1960s breeders in several programmes across several countries recommenced significant crossing with basic *S. spontaneum* clones for several reasons:

1. A concern about the small number of ancestral clones on which sugarcane breeding programmes were based compared with the great diversity of materials available: Arceneaux (1967) and Price (1967) both reviewed the derivation of modern sugarcane varieties and emphasised the limited numbers of ancestor clones. Most crosses made in breeding programmes throughout the world today are still based on a relatively small number of ancestors derived from the early interspecific hybrids. In Louisiana, a new strain of mosaic virus (later designated as strain H) was first acknowledged in the 1950s. When it began to infect varieties considered resistant to other strains of the disease, it highlighted the need for increased genetic diversity in the breeding programme and new sources of variation (Abbott 1961; Breaux and Fanguy 1967).
2. An awareness that there are many desirable traits in clones in germplasm collections (e.g. drought tolerance, waterlogging tolerance), not yet captured in commercial varieties.
3. Some unease because the rate of genetic gain was slowing, with one hypothesis for this being that it was related to the narrow genetic base of sugarcane breeding programmes.

By the 1970s, the so-called base broadening programmes were present in Barbados, Australia (Macknade), Taiwan, India, China, and the USA (Hawaii and Louisiana) (Heinz 1987). Subsequently programmes commenced in other countries such as Thailand and Brazil. The focus of most of these programmes was, and

continues to be, introgressing traits from *S. spontaneum* into commercial sugarcane varieties. Traits of interest from *S. spontaneum* include dense plant cane stands, profuse tillering, good ratooning ability, disease resistance, stem borer resistance, and stress tolerance (e.g. flood, salt, drought, cold) (Duncleman and Breaux 1970, 1972; Walker 1972; Heinz 1987). The approach taken by the programmes to identify *S. spontaneum* breeding clones varied, with the USDA-ARS-Houma, LA, choosing to heavily screen parental material before crossing and the germplasm enhancement programme in Barbados deliberately choosing not to select wild clones in order to maximise variability.

In most cases, the *S. spontaneum* is used as the male parent because of its heavy pollen production, its ability to self-pollinate, and its status as a noxious weed. By using the species as a male, breeders are able to ensure that resulting seedlings are not a result of self-pollination of the *S. spontaneum* parent. The original sugarcane × *S. spontaneum* (or other wild species) cross is referred to as the F_1 generation. Selected F_1 clones are backcrossed to commercial sugarcane genotypes (generally high sucrose) to obtain the first backcross generation (BC_1). Selected BC_1 clones are again backcrossed to sugarcane giving rise to the second backcross generation (BC_2), and an iterative process is continued (sometimes through the BC_4 generation or higher) until desired commercial parental clones are obtained.

Some successes (i.e. release of productive new commercial varieties) have arisen from introgression breeding programmes incorporating new germplasm since the 1960s, but overall, the success rate has been mixed and sometimes poor. Roach (1984, 1989), based partly on direct experience with the CSR programme in Australia, listed several reasons for the failure of new introgression programmes to provide more productive varieties than equivalent effort devoted to improved breeding pools. These reasons were largely related to inferior traits in the wild donor clones and difficulties in selecting and combining the appropriate desirable portions of both the wild type and the recurrent parents during subsequent selection cycles. Another problem noted was the lack of cytological or genetic information to confirm the hybrid nature of initial clones derived from interspecific hybridisation and selected for further crossing. Grassl (1963) also highlighted the challenges of obtaining synchronous flowering. However, both these issues have largely been overcome in recent decades with the use of DNA markers and photoperiod treatments. In particular, the use of DNA markers has been of greater importance as illustrated in examples reported by Cai et al. (2005) and Aitken et al. (2007a, b) in the identification of true hybrids.

The overall major challenge associated with introgression of basic germplasm into highly selected and commercially adapted germplasm in sugarcane breeding is the same as in other crops: that the basic germplasm brings with it many undesirable traits which need to be selected against between cycles of crossing back to the highly bred and commercially superior parental material, while at the same time desirable traits and genes from the wild donor may be diluted or lost with successive generations. In the case of sugarcane, the major undesirable trait introduced with the use of wild canes is low sucrose content. Following initial crosses made with wild clones (e.g. *S. spontaneum*), generally two or more backcrosses to elite commercial

type parents and lengthy (up to 7 or 8 years) intervening field evaluation and selection programmes are traditionally conducted before commercial type progenies are developed (Miller and Tai 1992). Therefore the process of introgression in sugarcane using conventional breeding procedures is relatively long and risky, and this deters investment.

Despite the challenges, some important successes have been achieved in introgression programmes initiated since the 1960s. One highly successful example is the development of numerous important cultivars in Australia within the BSES Limited breeding programme developed from the wild *S. spontaneum* clone "Mandalay". This clone was first collected by A.J. Mangelsdorf in 1929 (Heinz 1980) and was induced to flower in synchrony with commercial parents by CSR Limited in Australia in 1962. The flowers were crossed with POJ2878 in 1962 by BSES breeders, and selected progenies were crossed with other commercial varieties to produce a series of major cultivars for the Australian sugarcane industry. Another successful example is the use of the *S. spontaneum* cultivar US56-15-8, collected from northern Thailand, and leading to a range of cultivars in Louisiana, including the major cultivar LCP85-384 (Milligan et al. 1994; Arro et al. 2006).

A range of reports on the use of *S. spontaneum* since the 1960s are present in the literature. Some common findings of these programmes are as follows:

- Early generations of progeny following initial crosses between *S. spontaneum* and sugarcane (*S. officinarum* or commercial hybrids) are characterised by levels of sucrose content too low for commercial production and high fibre levels. These levels progressively and rapidly increase in subsequent crosses (e.g. Hsu and Shih 1989; Mullins 1988).
- Some early-generation progenies derived from *S. spontaneum* have provided good biomass yields, particularly in ratoon crops (e.g. Terajima et al. 2007; Wang et al. 2008), although it should be noted that some studies have involved small plots in which competition effects may be important in affecting cane yield.
- As noted by Roach (1984) much breeding work involving *S. spontaneum* following the 1960s was conducted under the vague objective of "base broadening". This objective in itself has little value and may not provide a focused basis for achieving practical commercial results.
- Some *S. spontaneum* clones provide good sources of resistance to diseases such as sugarcane mosaic (Koike 1980), red rot (Hale et al. 2010), and sugarcane yellow leaf virus (Costet et al. 2012) and pests (e.g. Jackson and Dunckelman 1974; White et al. 2011).
- Some *S. spontaneum* clones provide good sources of resistance to environmental stresses such as cold tolerance (e.g. Irvine 1966; Tai and Miller 1986; Miller et al. 2005) and waterlogging tolerance (e.g. Srinivasan and Batcha 1962; Sookasthan et al. 1992).
- Some contradictory results have been reported regarding the heritability of traits from *S. spontaneum* parental clones. Roach (1977) reported results showing that sucrose level in *S. spontaneum* clones was an important predictor of progeny performance when crossed with sugarcane (*S. spontaneum* or commercial type clones),

while cane yield was a moderate predictor. Wang et al. (2008) found performance of *S. spontaneum* parent clones to be a poor predictor of progeny performance for both sucrose content and yield, although it was high for stalk number and stalk weight independently.

- Although very preliminary, there are some signals that *S. spontaneum* clones sourced from the northern Thailand–Burma region could provide superior breeding material. Commercial successes in Australia and the USA have been obtained from clones selected in this region (as noted in references above). The impressive phenotypic characteristics of this material were highlighted by Sookasthan et al. (1992). Heinz (1980) noted the high yields of *S. spontaneum* clones collected from the northern tip of Thailand (19–20° latitude) and the apparent propensity to pass these favourable traits onto progeny. White et al. (2011) noted the significant contribution of *S. spontaneum* clones sourced from Thailand in sugarcane improvement.

14.4 Introgression of *Erianthus*

The genus *Erianthus* is related to *Saccharum* (sugarcane) and *Miscanthus* and is regarded by sugarcane breeders as a part of the *Saccharum* complex (Daniels and Roach 1987). The taxonomy of *Erianthus* is confusing, with some taxonomists regarding *Erianthus* as being within the genus *Saccharum*, although molecular studies indicate a relatively large difference with all other *Saccharum* species (Sobral et al. 1994; Hodkinson et al. 2002; Nair et al. 2005). There has been an interest among sugarcane breeders in utilising *Erianthus* in breeding programmes, particularly *Erianthus arundinaceus*, for many years. This is mainly due to the high level of vigour, drought and waterlogging resistance, good ratooning ability, and disease resistance attributed to this species. However, despite this reputation, it would appear that reports of well-controlled studies comparing performance of *Erianthus* with other *Saccharum* species and sugarcane cultivars are rare (Jackson and Henry 2011).

There have been several published reports of production of hybrids between *Saccharum* spp., especially *Saccharum officinarum* and sugarcane cultivars and *Erianthus arundinaceus* (D'Hont et al. 1995; Piperidis et al. 2000; Ram et al. 2001; Cai et al. 2005; Nair et al. 2006; Lalitha and Premachandran 2007), *Erianthus rockii* (Aitken et al. 2007a, b), and *Erianthus ravennae* (Janaki-Ammal 1941). We are aware of breeding programmes aiming to utilise *Erianthus* existing in a range of countries including China, India, the USA, Australia, and Thailand.

Several factors have limited the introgression of *Erianthus* in sugarcane breeding programmes to date, the major being the difficulty in producing fertile hybrids between sugarcane and *Erianthus*. This has been further complicated by difficulties in identifying true hybrids arising within seedlings from crosses between sugarcane (*Saccharum* spp.) and *Erianthus*. In many cases, putative hybrids have later been shown with DNA markers to be selfs or arising from pollen contamination (D'Hont et al. 1995, personal communication with sugarcane breeders). Further, while some

true hybrids have been produced, some breeders have reported difficulty in producing fertile crosses between these resulting hybrids and *Saccharum* (Piperidis et al. 2000). Difficulties in producing fertile hybrids, or any hybrids at all, may be attributed to the apparently relatively large genetic distance between *Saccharum* and *Erianthus*, even larger than for other genera such as *Miscanthus* (Sobral et al. 1994; Alix et al. 1998; Cai et al. 2005).

One successful example of progeny produced from hybrids between *Saccharum* and *Erianthus* was reported by Cai et al. (2005). However to date no commercial cultivars of sugarcane incorporating components of *Erianthus* have been reported to our knowledge.

Other factors suggested as potentially contributing to the lack of success of introgression of *Erianthus* genes into sugarcane breeding programmes include chromosome erosion during crossing and backcrossing and lack of recombination between the chromosomes of the two genera (D'Hont et al. 1995), although some recombinant chromosomes have been recently reported (Piperidis et al. 2012). Reduced transmission of chromosomes in crosses between *Saccharum* and *Erianthus* or hybrids and *Saccharum* was reported by D'Hont et al. (1995), Piperidis et al. (2000, 2010).

To date there have been few reports backed with statistically supported data demonstrating performance of *Saccharum*×*Erianthus* hybrids or their derivatives. Grassl (1972) reported vigorous and good-looking plants arising from a cross between *Erianthus kanashiroi* and *S. spontaneum*. Sugarcane clones produced by crossing an *Erianthus arundinaceus* clone with a *S. officinarum* clone were also reported by Ram et al. (2001) as having superior low temperature tolerance and red rot resistance.

14.5 Introgression of Other Species

Although its inclusion as part of the *Saccharum* complex has been debated, *Miscanthus* is a genus of interest to sugarcane breeders. The *Miscanthus* genus is distributed across Tahiti, Indonesia, China, Siberia, Japan, India, southern Africa, and Nepal (Adati and Shiotani 1962; Hodkinson et al. 2002). The genus is found in elevations ranging from sea level to 3,300 m in Taiwan (Lo et al. 1978) but is mainly found in upper elevations (1,500–2,500 m) (Paijmans 1976). Much like *S. spontaneum*, *Miscanthus* can be found growing in diverse environments including rocky, stony, dry, or wet and in full sun or shade (Lo and Su 1968). *Miscanthus sacchariflorus* is thought to be one of the species involved in the evolution of *S. sinense* (Grassl 1974), and phylogenetic analysis of cytoplasmic DNA suggests that *Miscanthus* and *Saccharum* hybridise naturally (Sobral et al. 1994). Traits of interest to sugarcane breeders from this genus include cold tolerance, downy mildew resistance, drought resistance, and smut resistance (Lo et al. 1978).

While hybrids with the genus are not common, some successful attempts have been documented. Tai et al. (1991) evaluated juice quality traits of hybrid and

backcross progeny of *Saccharum* and *Miscanthus* and found that mean sucrose content of F_2 and BC_1 progenies was higher than that of the F_1 hybrids, but stalk diameter remained small. Burner (1997) describes an $n+2n$ transmission when sugarcane was crossed to *M. sinensis* and $n+n$ transmission when crossed with *M. japonicas*. While no DNA-based markers were used in this study, chromosome transmission was documented, lending credence to the claim that successful hybridisations were made between *Saccharum* and *Miscanthus*.

Breeders in Taiwan have had an interest in breeding with *Miscanthus* since the 1950s. In 1953, a study was published on a cross between POJ2755 and *Miscanthus floridulus* (Chen 1953). One hundred and twenty-nine clones in their collection were screened for resistance to smut and downy mildew, and over 80 % were resistant to downy mildew. All these clones were resistant to smut (Lo et al. 1980). Downy mildew-resistant clones were used as males and crossed to commercial hybrids, resulting in 21 % of progeny that morphologically resembled *Miscanthus*. Selected progenies were downy mildew resistant, and a majority were highly resistant to smut. They had a brix that was higher than the *Miscanthus* parent (approaching that of sugarcane), a stalk diameter intermediate between the two parents, and populations that were greater than the sugarcane parent (Chen et al. 1980, 1982, 1983; Shen et al. 1981). In 1983, the research group reported that F_1 hybrids between sugarcane and *M. sinensis* or *M. floridulus* resulted in progeny with average pith, diameter, sucrose content, and tillering somewhere between the two parents. These were assumed to be true hybrids because of chromosome numbers ranging from $2n=70$ to 100 with irregular meiosis (Chen et al. 1983).

The recent emphasis on bioenergy production has corresponded with a renewed interest with intercrossing *Saccharum* and *Miscanthus*. The USDA-ARS in Houma, LA, has bred with *Miscanthus* in the past and has a few verified hybrids (unpublished). Recently, Texas A&M University has reported that hybrids between the two genera, named "Miscanes", have shown promise as donors for drought and cold tolerance. The group has verified the hybrid nature of the clones and continues to work with breeding them for bioenergy production (Park et al. 2011).

There have been several reports of hybridisation between sugarcane and bamboo, although hybridisation attempts resulted in very few viable seeds. Rao et al. (1967) reported the generation of four hybrid seeds from 960 crosses between *Bambusa arundinacea* (*B. bambos*) and *Saccharum*. When *Bambusa* was used as the male parent, no hybrids were obtained; however, when it was emasculated and used as the female parent, two seeds were produced from *S. spontaneum* and two from *S. robustum*, while the seed from the *S. spontaneum* only germinated.

Sorghum is another genera of interest to sugarcane breeders because it possesses drought tolerance, is widely adapted, and offers the potential to develop a hybrid crop that can be propagated through seed. Several attempts at hybridisation with *Sorghum* have been made over the last century. In 1930, Thomas and Venkatraman reported successful hybridisation between sugarcane and *Sorghum dura* Stapf, and in 1935, Bourne reported hybridisations between sugarcane and sweet sorghum, *Holcus sorghum* L. var. *saccharatus* (L.) Bailey. In both cases, hybrids were reported to be dwarf, with some albino types, and had little commercialisation potential.

Overall, 37 % of the 345 hybrids produced by Bourne survived, with only 3 % displaying enough vigour for further evaluation. The hybrids produced by Venkatram were said to mature in 5–6 months in comparison to the 9–10 months required for cane and despite their low yields were "high in sugar". (Brandes 1935). Another hybridisation between *Saccharum* and *Sorghum* was reported in 1999, but like previous attempts, recovery of viable seedlings was low, with only five seedlings recovered from 3,670 pollinated florets (Nair 1999).

Hybridisation between the two genera was reported recently (Hodnett et al. 2010). While past attempts at hybridisation met with poor seed set, a new approach was taken using an inbred line of *Sorghum bicolor* which was homozygous for the mutant *iap* (inhibition of alien pollen). The *iap* trait removes the reproductive isolation between sorghum and closely related taxa allowing for easy production of interspecific hybrids. Through the use of Tx3361 (the line containing the *iap* trait) as the male sterile female parent, 14,141 hybrid seeds were produced from 252 *Sorghum bicolour*×*Saccharum* crosses. Embryo rescue was used on the pollinated seed resulting in a seedling recovery rate of 33 %. Attempts to backcross the hybrids to sorghum have not been successful.

14.6 Transfer of Genes Through Genetic Engineering

As discussed above, sexual transmission of genes and traits is limited to relatively close relatives and members of wider Andropogoneae where sexual compatibility is allowed. However, the ability to introduce and express alien genetic materials into sugarcane by artificial methods allows possibilities of gene transfer from any other organism, even those from other kingdoms. The first demonstration of this process, called transformation or more broadly "genetic engineering", for sugarcane was the introduction of a bacterial gene conferring resistance to an antibiotic kanamycin into sugarcane protoplasts (Chen et al. 1987) by osmotic shock. Additional methods of genetic engineering used since then include electroporation (Chowdhury and Vasil 1992), biolistics (Franks and Birch 1991), and the soil bacterium, *Agrobacterium* (Arencibia et al. 1998).

The delivery and subsequent integration of the introduced genetic material is only the first step: to make introgression of alien genes useful, plants expressing them at the desired level need to be regenerated from the cells/tissues into which they were introduced. This has been done successfully in a variety of targets ranging from protoplasts to cells to different tissue types (Lakshmanan et al. 2005). Unlike the natural development of plants from axillary buds, regeneration of plants from a dedifferentiated transformed cell or tissue in an artificial sterile condition meant that their phenotype, including agronomic characteristics and yield, may vary from the mother plant (Lakshmanan 2006). Hence, identifying desirable genetically modified lines from a large population of transgenic plants is a critical step in crop genetic engineering. This involves not only the assessment of agronomics and yield of

genetically engineered lines but also the stable expression of introduced alien gene/traits(s) in different production environments and transmission to progenies in successive generations. This has been demonstrated by engineering herbicide tolerance and virus resistance traits simultaneously in sugarcane (Butterfield et al. 2002). However, it is important to note that expression of alien genes introduced transgenically may become silenced when grown in the field following successive vegetative propagation (Basnayake et al. 2012).

There are several types of alien genetic elements that have been introduced into sugarcane through transformation. Firstly, and most importantly, there are coding regions of genes, which confer the target trait/phenotype such as pest and disease resistance and herbicide tolerance. To make the coding region of gene express a desired phenotype the genetic message it carries will be transcribed into the messenger molecule RNA. Gene transcription is regulated by a promoter and terminator and sometimes enhancers. These regulatory elements also can be of foreign origin, and numerous such examples exist in sugarcane (Lakshmanan et al. 2005), starting from the very first creation of a genetically engineered sugarcane cell (Chen et al. 1987).

Since gene transfer through transformation is largely a mechanical process, both alien and native genes and/or its regulators in its original or modified forms can be introduced (Beyene et al. 2011). Also, in the same sugarcane line multiple genes controlled by genetic elements of diverse origin can be introduced. This becomes a necessity when a new metabolic pathway involving multiple genes needed for a target product is engineered into plants. Despite its technical complexity this has been achieved in sugarcane engineered to produce bioplastics (Mcqualter et al. 2004). More recently microRNAs controlling specific traits are being manipulated to modify phenotypes by altering the level of specific gene expression. For example, sugarcane microRNAs have been successfully used in tobacco (Begcy et al. 2012) and in sugarcane (Jung et al. 2012). With the explosion of genomics research in sugarcane and other crops application of native and alien microRNAs may become a significant tool for genetic modification in sugarcane.

The fact that the fundamental features of genetic elements are the same across all organisms, genes from any source can be introduced into sugarcane. Table 14.1 shows examples from various phyla from which DNA has been introduced into sugarcane. However, sometimes the coding regions of genes are redesigned to optimise its expression in other species. The most common changes have been (1) codon optimisation to produce an enzyme in the correct configuration and (2) use of eukaryotic introns in prokaryotic genes to affect the expression in a eukaryote-like sugarcane.

DNA sequences originally isolated from sugarcane have been transformed back into sugarcane such as genes (e.g. polyphenol oxidase, Vickers et al. 2005a, b), promoters (Mudge et al. 2009), and targeting sequences (Jackson et al. 2007), but this is referred to as *cis*-genics and is not discussed here further.

Several examples of sugarcane plants engineered using alien genes are described below:

Table 14.1 Sources of some of the genes introduced into sugarcane through biotechnological methods

Phyla	Species, DNA, and trait	References
Virus	Sugarcane mosaic virus, coat protein gene (resistance)	Joyce et al. (1998)
	SrMv (sorghum mosaic virus) (resistance)	Ingelbrecht et al. (1999)
	Sugarcane yellow leaf virus (resistance)	Rangel et al. (2003)
	Fiji disease virus (resistance)	Mcqualter et al. (2001)
	Cauliflower mosaic virus promoter (gene expression)	Gallomeagher and Irvine (1993)
	Cauliflower mosaic virus terminator (gene expression enhancement)	Beyene et al. (2011)
	Cavendish banana streak badnavirus promoter (gene expression)	Petrasovits et al. (2012)
Bacteria	*Pseudomonas mesoacidophila* MX-45, trehalulose synthase (alternative sugar)	Hamerli and Birch (2011)
	Pantoea dispersa, albicidin dehydrogenase (resistance to leaf scald)	Zhang et al. (1999)
	Pantoea dispersa UQ68J isomaltulose synthase (alternative sugar)	Basnayake et al. (2012)
	Ralstonia eutropha phaA, *phaB*, *phaC* (polyhydroxybutyrate)	Petrasovits et al. (2007)
	Agrobacterium tumefaciens nopaline synthase terminator (gene expression)	Petrasovits et al. (2007)
Fungi	*Grifola frondosa*, trehalose synthase gene (drought tolerance)	Zhang et al. (2006)
Animals	*Aequorea victoria,* green fluorescent protein (selectable marker)	Elliott et al. (1999)
Human	Human-granulocyte macrophage colony-stimulating factor gene (pharmaceutical)	Wang et al. (2005)
Dicotyledons	Soybean proteinase inhibitor genes (insect resistance)	Falco and Silva-Filha (2003)
Monocotyledons	Maize ubiquitin promoter (gene expression)	Hamerli and Birch (2011)
	Maize chlorophyll a/b-binding protein	Petrasovits et al. (2012)
	Rice ubiquitin promoter (gene expression)	Petrasovits et al. (2012)

14.6.1 Protecting Against Sugarcane Leaf Scald

Leaf scald is a major bacterial disease caused by *Xanthomonas albilineans*, which produces the toxin albicidin. Engineered resistance to leaf scald was one of the first significant examples of exploiting alien genes for a commercially important trait (Zhang et al. 1999). Transgenic sugarcane plants that express an albicidin-detoxifying gene (*albD*) cloned from the bacterium *Pantoea dispersa* which is used as a biocontrol against leaf scald disease did not develop disease symptoms in inoculated leaves, whereas all non-transgenic control plants developed severe symptoms. Expression of *albD* gene also protected plants against systemic multiplication of the pathogen proving that a single gene-based detoxification strategy alone *is sufficient to confer resistance to both disease symptoms and control of pathogen in the host*.

14.6.2 Production of Alternative Sugars in Sugarcane

Use of sugarcane as a biofactory for alternative sugars attracted substantial interest in the past decade. As an example Basnayake et al. (2012) were successful to express isomaltulose synthase (IMS) gene in stem parenchyma vacuole of sugarcane in order to produce isomaltulose (IM), a low GI alternative sugar. Transgenic sugarcane plants of seven Australian cultivars expressing IMS gene when grown in the field accumulated IM up to 33 % of total sugar. However, a concomitant decrease in sucrose concentration resulted in no change in total sugar content in these IMS lines. Several cycles of field propagation and careful selection were needed to identify clones that yielded similar to the recipient non-transgenic lines. Clones with no apparent adverse effect of IM accumulation on growth and germination of setts were identified in the test population. However, there was some inconsistency in IM production in vegetatively propagated field-grown transgenic lines. Despite this observation, the results in general indicated good potential to develop sugarcane for commercial scale production of IM.

14.6.3 Weed and Mosaic Virus Control in One Shot

Transgenic sugarcane plants resistant to herbicide bialaphos and sorghum mosaic virus (SrMV) were produced by introducing alien genes *bar* (herbicide resistance) and *hut* (SrMV resistance) (Ingelbrecht et al. 1999). These lines were crossed with non-transgenic sugarcane varieties to study the segregation of the transgenes and trait expression in the progeny. Both transgenes were integrated in the genome as a linked insertion in one locus or they occurred in two independent, unlinked loci. Analysis of progeny of parent unlinked independent insertions indicated rearrangements in both loci. Most transgenic progenies containing the *bar* gene showed resistance to herbicide, while a high proportion of progenies were susceptible to SrMV. This pioneering study on transgene segregation in sugarcane demonstrated the viability of transgenic sugarcane parents in breeding programmes.

Whilst some plants have been tested in field trials, development of commercial sugarcane varieties from transgenic technology has yet to be realised. Several specific steps including additional technical, regulatory, and commercial aspects which are generally not part of a research activity need to be addressed to develop genetically modified (GM) commercial sugarcane cultivars.

The first technical issue is to reduce the number of transgene insertions. Ideally a potential commercial GM clone will have an intact single-copy transgene insertion event for simple integration during sexual transmission to other genetic backgrounds. The stability of trait expression across multiple vegetative generations under different crop production environments is another major consideration for commercial GM crop development. Further, transgene introduction must not have a negative impact on cane or sugar yield: early field trials indicated that this could be the case (Vickers et al. 2005a, b). Additionally, introduction of the alien gene should

not predispose the host to susceptibility to diseases and pests. The transgene should also perform consistently in different genetic backgrounds, a key requirement for successful introgression of the transgenic trait through breeding.

Regulatory agencies assess GM plants and the food originating from them for a range of potential hazards relating to the environment and human health. For the environment, the ability of the transgene to increase the weediness of the altered plant or any sexually compatible species is assessed as is the potential effect of the transgene product on the organisms it may come in contact with. These impacts are assessed on a case-by-case basis, but information required to define the baseline biology of sugarcane to assist assessment has been receiving attention (Bonnett et al. 2008; Office of Gene technology Regulator, Australia (OGTR) 2011; Cheavegatti-Gianotto et al. 2011). An Organisation for Economic Co-Operation and Development (OECD) consensus document on GM sugarcane is also being prepared. For regulation in food, a common practice is to assess the substantive equivalence of the new plant compared to the existing ones, and a recent document from the OECD has suggested about what components of sugarcane modified with alien genes should be tested for comparison (OECD 2011). In addition, the potential for the inserted DNA to produce toxins or allergens is assessed, and sometimes feeding studies are also conducted.

Whilst at the time of writing no sugarcane with an alien gene transferred by molecular techniques has yet been commercialised, it is likely to occur within the next few years. Significant investments are being made in sugarcane by companies that have brought other genetically modified crops to the market (Bonnett et al. 2010) which will increase the effort in commercialisation of sugarcane with alien genes transferred by molecular techniques. The high cost of regulating each individual event and the long time to breed new sugarcane cultivars will see increased discussion of whether regulation can be conducted on an individual gene construct basis (allowing different events arising from the use of the same assembly of alien gene elements into different background cultivars).

14.7 Conclusions and Future Prospects

The development of modern sugarcane cultivars has provided a great example of the successful introgression of genes from wild germplasm for enhancement of genetic gains from breeding. The introgression of parts of the wild cane *S. spontaneum* genome into the *S. officinarum* genome in the early 1900s provided improved disease resistance, better adaptation to environmental stresses, and much improved ratooning performance and had enormous impact on lowering the cost of sugar production worldwide thereafter.

Following the first interspecific hybrids, sugarcane breeding programmes continued to make large gains from this initial introgression for several decades, illustrated by clear superiority of successions of new varieties in many industries until at least the

1950s. However, based on anecdotal evidence it appears that after several decades of exploiting the newly introgressed materials, rates of genetic gain gradually slowed. This prompted concerns expressed by a range of sugarcane breeders around the 1960s and stimulated efforts to introgress more genetic diversity from wild canes, particularly *S. spontaneum*, into sugarcane breeding programmes. While there have been some successes from these more recent efforts, the gains have clearly not been as large as the initial introgression breeding, and there have been many cases where little or no commercial success has arisen from significant efforts.

Introgression of new wild cane genomes which is occurring in a range of sugarcane breeding programmes around the world is expected to continue into the future. Based on current reports this effort will probably provide for further incremental gains in profitability. However, because of the need to obtain high sugar content for commercial cultivars, and the very low sucrose content in wild canes, several cycles of backcrossing and selection are required, and this will continue to make investment in conventional approaches to introgression breeding lengthy, costly, difficult, and risky.

Berding and Roach (1987) and Roach (1992) amongst many others have lamented the lack of characterisation of clones in sugarcane-related germplasm collections and pointed to this as limiting wider and more effective use of material in collections. While some characterisation of clones in various collections has been reported (Roach 1986; Tai and Miller 1988; Balakrishnan et al. 2000), it is also questionable as to whether characterisation for some traits provides a useful indicator of breeding value. One line of argument is that for complex traits like yield wild species may contain mostly inferior traits and alleles but that at a smaller number of loci, some alleles with more favourable effects than in existing commercial materials may exist (Tanksley and McCouch 1997). The key challenge to the breeder is therefore retaining the favourable alleles while eliminating the rest during the backcrossing cycles. Identification of favourable alleles amongst unfavourable ones may benefit from DNA markers for QTL analysis (see below). This argument is consistent with some reports of poor-performing wild clones proving to produce the best progeny in two or more generations (Rao and Martin-Gardiner 2000).

Two developments in the future may improve contributions from introgression of wild germplasm in sugarcane breeding. Firstly, the likely increased value of the fibre component in sugarcane for energy production (electricity or biofuel) in future may tilt optimal selection indices in sugarcane breeding programmes slightly away from the current very high weighting to sucrose content and more towards total biomass production and fibre content. If this occurs then commercial cultivars with a higher proportion of the wild *S. spontaneum* genome may be possible, meaning that less cycles of backcrossing may be required and early-generation clones (F_1 or BC_1) or crosses among such clones may be commercially viable. Such a change would increase the accessibility and use of wild clones in sugarcane breeding programmes.

Secondly, as with all other crops, the application of DNA markers may also provide ways to better use wild germplasm in breeding programmes. Undertaking QTL

analysis in advanced backcrosses may provide a way to effectively identify the wanted and unwanted parts of the wild genome. Markers closely linked to the desirable and undesirable genome components could be selected for or against during subsequent breeding efforts.

The use of DNA markers for assisting in introgression breeding however provides some challenges in practical implementation, particularly in costs and time. Undertaking QTL analysis to detect markers linked to traits at low false discovery rates for most quantitative traits usually requires field phenotyping and genotyping of large (>500) populations. Generation of advanced backcross populations in sugarcane also usually takes several years. However, despite these costs such approaches may still provide an effective way to utilise wild genomes in sugarcane breeding more effectively than conventional approaches.

The large genome size of sugarcane and the high polyploidy add additional challenges to the application of DNA markers in sugarcane which need careful consideration. For example if the donor genome (e.g. *S. spontaneum*) has a high ploidy level, progeny in first or second backcross generations may not segregate in terms of the presence versus absence of chromosomes from a homology group. Such populations therefore may only provide analysis of variation due to alternative alleles from the donor germplasm, rather than test for the presence versus absence of any donor germplasm alleles at different loci. The large genome size of sugarcane also means that a large number (e.g. >3,000) of markers may be required to obtain comprehensive genome coverage, and with many marker systems used (e.g. AFLPs) this is very expensive. The development of newer marker systems such as SNP chips may help address this issue in the future in sugarcane. However obtaining sufficient phenotype data to undertake powerful QTL mapping for most important traits (e.g. sugar content and cane yield) will remain slow and expensive without some high-throughput technologies.

Technological advancements in GM sugarcane research have also been impressive with a variety of methods to introduce and express genes in sugarcane now available. However, as in many other crops, a major hurdle is recalcitrance of sugarcane genotypes. More research effort is needed to tackle this issue. Recently, the value of linearised minimal gene vectors carrying only the expression cassette for GM sugarcane research was recognised (Jackson et al. 2013), and it is expected to become a routine technology for sugarcane transformation. Development of synthetic mini chromosomes that offer the ability to target transgenes to a defined insertion position for predictable expression (Birchler et al. 2010) is likely to make a significant impact in GM technology especially in polyploids. Sugarcane, touted as a viable energy crop, is attracting large investments in developing commercial GM crops in various countries. The target of alien (GM) traits in the near to medium term will be those that are not readily available for conventional breeding, as occurred in other crops. These include herbicide tolerance, pest resistance (e.g. stem borer resistance), and production of foreign compounds (e.g. alternative sugars). However, it must be stressed that the high regulatory costs and intellectual property restrictions may restrict the development of commercial GM sugarcane to mostly large multinational companies for the foreseeable future.

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Chapter 15
Tomato

Pritam Kalia and Muthukumar Palanisamy

Abstract Tomato (*Solanum lycopersicum L.*) is the second most consumed vegetable after potato and finds several direct and indirect uses in human diet, providing significant sources of vitamins A and C, essential minerals, phenolic antioxidants and several other nutrients. Important breeding objectives in tomato include increasing fruit yield, improving sensory and nutritional quality and adaptation to biotic and abiotic stresses. Cultivated species of tomato has relatively little genetic variability due to an inbreeding mating system as well as several population bottlenecks. Nonetheless, during the past more than 70 years, wild species have been utilised to introgress desirable characters in cultivated tomato and widen its genetic base. Consequently, tomato has become one of the crops which have been benefitted most from alien introgressions. Among wild species, *S. chilense*, *S. peruvianum*, *S. habrochaites* and *S. pimpinellifolium* have been observed to be the richest sources of desirable alien genes and have been extensively used in hybridisation programmes. While the earlier introgressions were achieved mainly through conventional backcrossing, of late, these have been aided by genomic tools. This chapter reviews such significant developments in tomato improvement through alien gene transfer and analyses their impacts on improving tomato productivity, nutritional quality and adaptation to different biotic and abiotic stresses.

Keywords Alien introgression • QTLs • Biotic stresses • Abiotic stresses • *Solanum lycopersicum* • *S. pimpinellifolium*

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15.1 Introduction

Tomato (*Solanum lycopersicum L.*) is an important vegetable crop consumed and grown the world over in tropical to temperate environments. It is the second most consumed vegetable after potato and unquestionably the most popular garden crop suitable for a diverse and balanced diet. Nutritionally, tomato does not rank high as one medium fresh tomato (135 g) provides 47 % RDA of vitamin C, 22 % RDA vitamin A and 25 calories. However, by virtue of volume consumed, it contributes significantly to the dietary intake of vitamins A and C as well as essential minerals and other nutrients. It ranks first among all fruits and vegetables as a source of vitamins, minerals and phenolic antioxidants. Also, fresh and processed tomatoes are the richest sources of the antioxidant lycopene, a powerful anticancer bioactive compound. In addition to tomatoes that are eaten raw or in cooked form, a variety of processed products such as paste, whole peeled tomatoes, diced products and various forms of juices, sauces and soups have gained significant acceptance. Although a tropical plant, tomato is grown in almost every corner of the world from the tropics to within a few degrees of the Arctic Circle. It is grown in greenhouses where outdoor production is restricted due to cool temperatures. Major tomato-producing countries in descending orders include China, the USA, India, Turkey, Egypt and Italy (http://faostat.fao.org). Other leading countries include Spain, Brazil, Iran, Mexico, Greece and Russia.

It has been shown that several biotic and abiotic stresses affect the production and productivity of tomato and these yield constraints are a major threat to the growers in raising a profitable crop. Wild species are important reservoir of useful genes for resistance to biotic and abiotic stresses. Studies have shown that the introgression of alien genes has taken place naturally, which led to evolution of cultivated species, *S. lycopersicum*. Latent genetic variation has been identified in the germ plasm collections, which can indicate novel genetic variation for exploitation by end users or, conversely, reveal unfavourable and hence undesirable alleles with respect to crop improvement (Rick 1958; Rieseberg et al. 2000). Therefore, these alien genes for agronomically important traits are important resources for developing improved varieties/hybrids suitable for various stress situations in tomato. Wild species have been used extensively in breeding programmes for development of such improved cultivars that made less input intensive and pesticide-free cultivation of tomato over a long period. In this chapter, we discuss the achievements and impacts of alien gene transfer made from transfer of alien genes from wild species using traditional and genomic tools and techniques.

15.2 Genetic Resources and Their Maintenance

The plant group *Solanum* sect. *Lycopersicon* consists of 13 closely related species or subspecies: the cultivated tomato, *Solanum lycopersicum* (formerly *Lycopersicon esculentum*), which includes the domesticated tomato and wild or weedy forms of the cherry tomato (*S. lycopersicum* "*cerasiforme*") (Peralta et al. 2008) and the wild

species, namely, *Solanum arcanum*, *S. cheesmaniae*, *S. chilense*, *S. chmielewskii*, *S. corneliomulleri*, *S. galapagense*, *S. habrochaites*, *S. huaylasense*, *S. neorickii*, *S. pennellii*, *S. peruvianum* and *S. pimpinellifolium*. Four species have been segregated from the green-fruited species, *S. peruvianum*; two of them *S. arcanum* and *S. huaylasense* have been described as new species (Peralta et al. 2005) from Peru, while the other two are *S. peruvianum* and *S. corneliomulleri*. In addition, *S. galapagense*, another yellow- to orange-fruited species, was segregated from *S. cheesmaniae*; both species are endemic to the Galapagos Islands. All members of section *Lycopersicon* are closely related diploid species ($2n=24$) and are characterised by a high degree of genomic synteny and are to some degree intercrossable.

It is estimated that over 62,800 accessions of the cultivated and wild species of tomato (mostly *L. esculentum* accessions) are maintained in gene banks around the world, including those in the Asian Vegetable Research and Development Center (AVRDC) at Tainan, Taiwan; the United States Department of Agriculture (USDA); Plant Genetic Resources Unit at Geneva (PGRU), NY, USA; the CM Rick Tomato Genetics Resource Center (TGRC), University of California, Davis, California, USA; and the National Bureau of Plant Genetic Resources (NBPGR), Indian Council of Agricultural Research, New Delhi, India. The TGRC (http://tgrc.ucdavis.edu) is known to maintain the largest collection of tomato wild species, while PGRU has a large collection of open-pollinated cultivars. Good collections of tomato germplasm are also maintained in the Netherlands (IVT), Russia (VIR), Japan (NIAS), Peru (DHUNA) and Cuba (INIFAT). In addition to the wild and cultivated accessions, there are thousands of tomato monogenic stocks and mutants that have been phenotypically characterised and catalogued (http://tgrc.ucdavis.edu; http://www.zamir.sgn.cornell.edu).

15.3 Genetic Variability

Depending on the type of use, different breeding objectives are pursued, which include improved yield, sensory and nutritional quality as well as adaptation to biotic and abiotic stresses (Rick 1988, 1990). Genetic improvement in tomato needs to rely on sufficient genetic diversity in order to be able to satisfy current and future breeding challenges. Cultivated tomato germplasm, however, has relatively limited genetic variation, resulting from its inbreeding mating system associated with severe genetic bottlenecks that are postulated to have occurred prior to, during and after the domestication process (Rick 1987). This limited variability in cultivated tomato could be largely due to founder event as well as due to natural and artificial selections that occurred during domestication and evolution of modern cultivars. For example, tomatoes that were first introduced to Europe by Spanish explorers furnished the entire genetic base for the modern cultivars, and consequently the current European and US cultivars are highly similar to each other. It is estimated that only ~5 % of the total genetic variation within *Lycopersicon* can be found within *L. esculentum* and genes for many desirable agricultural characteristics do not exist

in this species. In contrast, tomato wild species possess rich genetic variation and are potential sources for the improvement of many economically important traits (Rick 1987). It has been estimated that members of *Solanum* sect. *Lycopersicon* have been evolved seven million years ago (MYA) along with the other taxa related to sects. *juglandifolia* and *lycopersicoides*. These taxa are adapted to a wide variety of environmental conditions, which correspond to a wide range of variation in terms of morphology, physiology, mating system, and biochemical characteristics (Robertson and Labate 2007). Therefore, related wild tomato species have been shown to be rich source of desirable genes and characteristics for crop improvement. Many agriculturally important traits can be found in the potentially useful tomato wild accessions stored in gene banks; nonetheless, breeders have so far been unable to fully exploit this rich reservoir (Robertson and Labate 2007).

15.4 Alien Gene Transfer

Exchange of genetic information from the wild germplasm to cultivated species has taken place probably after 1940 when renowned geneticist and plant breeder Charlie Rick (University of California, Davis) observed a wild array of novel genetic variation in the offspring derived from crosses between wild and cultivated species. This has led to great utilisation of the favourable attributes hidden in tomato exotics through interspecific hybridisation in the twentieth century. As the result, during the past more than 70 years, wild species of tomato are being utilised in breeding programmes to improve the cultivated tomato, and source of disease resistance in most of the commercial cultivars has been derived from the related wild species. In fact, the gains through alien introgressions in tomato have been so huge that the cultivated tomato has become a prime example of crop plants that has benefited significantly from exotic germ plasm introgression. Recent advancements in molecular markers and marker-assisted selection (MAS) technology are expected to make tomato improvement via introgression from wild species more feasible.

15.4.1 Biotic Stresses

Wild species are the primary source of resistance for modern tomato varieties and hybrids, and resistance for more than 40 major diseases was discovered in wild relatives of tomato. Genetic improvement for developing disease resistance has taken place for at least 20 diseases in tomato using wild species (Ji et al. 2007a). Desirable alien genes for resistance to diseases and insect-pests have been transferred in cultivated background using conventional breeding programme as well as aided by recent molecular marker tools and techniques. *S. chilense*, *S. peruvianum*, *S. habrochaites* and *S. pimpinellifolium* (Fig. 15.1) have proved to be the richest sources of resistance genes (Foolad et al. 2003; Foolad and Sharma 2005; Laterrot 2000; Scott and Gardner 2007).

Fig. 15.1 Most frequently used wild species of tomato for alien gene transfer in cultivated background (**a**) *S. habrochaites*, (**b**) *S. peruvianum* and (**c**) *S. pimpinellifolium*

15.4.1.1 Disease Resistance

Based on inheritance studies, both "qualitative disease resistance" and "quantitative disease resistance" (QDR) have been observed to control disease resistance in tomato. Resistance genes that confer qualitative effects have been designated as R-genes, while loci or genes that confer QDR have been known as "QRLs" (quantitative resistance loci). R-gene often confers complete resistance against a specific race of pathogen. These R-genes have been identified in the background of wild species in tomato, which have been mapped using recent molecular tools (Table 15.1). Several resources and molecular approaches have been developed to fully exploit genetic potential in tomato breeding.

Actually, major resistance genes control QDR. These major genes exhibit a discrete (categorical) distribution of disease phenotypes in segregating population, and one or a few genes are responsible to control the QDR (Michelmore 1995). QDR tends to be more durable than typical R-gene-mediated resistance (Young 1996). Since QDR is controlled by multiple genes with partial and inconsistent effects, pathogen variants that overcome QRLs gain only a marginal advantage. QRL is conditioned by genes that regulate the morphological and developmental phenotypes representing mutations or different alleles of genes involved in basal defence. These are the components of chemical warfare and unique set of previously unidentified genes (Poland et al. 2009). Some QRLs have been reported to be coincident (i.e. have similar or identical linkage map positions) with major R-genes for disease resistances in various crop plants. Resistance controlled by quantitative resistant loci (QRLs) has been reported in wild tomato species (Table 15.2). Quantitative resistance is often non-race specific and more durable than qualitative resistance governed by R-genes (Palloix et al. 2009).

Powdery Mildew Resistance

Tomato powdery mildew caused by the fungus *Oidium neolycopersici* has become a serious disease in the Northern Hemisphere, especially in protected tomato cultivation. Resistance to *O. neolycopersici* in tomato has been observed as both monogenic and polygenic. The monogenic resistance is reported to be controlled by dominant and recessive genes. Three dominant genes (*Ol-1*, *Ol-3*, *Ol-6*) have been identified, which have been shown to be associated with a hypersensitive response (HR) (Bai et al. 2005; Li et al. 2007). These genes originated from different accessions of wild species including *S. habrochaites* (G11257, G11290, G11560, G11606 = CPRO742208, Lindhout et al. 1994) and *S. peruvianum*. The alien genes identified in the wild accessions of *S. habrochaites* have been mapped using molecular markers for their further precise transfer in the background of cultivated species (Van der Beek et al. 1994; Huang et al. 2000b; Bai et al. 2005; De Giovanni et al. 2004). A wild accession LA 2172 of *S. arcanum* showed the complete immune response. The dominant gene *Ol-4* was identified to be responsible for this complete resistance, which was mapped on tomato chromosome 6 (Bai et al. 2004, 2005).

Table 15.1 Resistance genes identified for various diseases of tomato in wild genetic background

Source of resistance	Disease	Accession number	Resistant genes/QTLs	References
S. arcanum	Powdery mildew	LA 2172	*Ol-4*	Bai et al. (2004, 2005)
S. pennellii	Fusarium wilt	LA0716	*I-1*	Sarfatti et al. (1991); Scott et al. (2004)
		LA0716 and PI414773	*I-3*	Bournival et al. (1989, 1990); Hemming et al. (2004); Lim et al. (2008)
		LA0716	*I-5*, *I-6*	Sela-Buurlage et al. (2001)
S. peruvianum	Tomato spotted wilt virus	–	*Sw-1a*	Finlay (1953); Stevens et al. (1992)
		–	*Sw-5*	Boiteux and Giordano (1992); Stevens et al. (1995); Brommonschenkel et al. (2000)
	Tomato yellow leaf curl virus	–	*Ty-5 (TY-172)*	Anbinder et al. (2009)
S. pimpinellifolium	Fusarium wilt	PI 79532	*I*	Paddock (1950); Scott et al. (2004)
	Fusarium wilt	PI 126915	*I-2* complex locus	Laterrot (1976); Sela-Buurlage et al. (2001a, b)
	Tobacco mosaic virus	PI 128650	*Tm2a (Tm-2^2) and Tm-2*	Lanfermeijer et al. (2003, 2005)
	Bacterial speck	–	*Pto* + *Prf*	Martin et al. (1991, 1993, 1995)
	Late blight	WVa700	*Ph-2*	Moreau et al. (1998)
		L3708	*Ph-3*	Chunwongse et al. (2002)
	Leaf mould	L3708	*CF-2*	Dixon et al. (1996); van der Beek et al. (1992)
		PI 126915	*CF-9*	Jones et al. (1993); Balint-Kurti et al. (1994); Parniske et al. (1997); Rivas and Thomas (2005)
		PI 126947	*Cf-ECP2*, *Cf-ECP3*	Haanstra et al. (1999)
		CGN15529	*Cf-ECP 5*	Haanstra (2000)
		LA1547, LA1683	*Cf-ECP1*, *Cf-ECP 4*	Soumpourou et al. (2007)

(continued)

Table 15.1 (continued)

Source of resistance	Disease	Accession number	Resistant genes/QTLs	References
S. chilense	Tomato yellow leaf curl virus	LA 1969	*Ty-1*	Michelson et al. (1994); Zamir et al. (1994)
		LA 1969, LA 1932	*Ty-3*	Ji et al. (2007a, b)
		LA 2779, *LA 1932*	*Ty-4*	Ji et al. (2009a)
	Tomato spotted wilt virus	LA1938	*Sw-7*	Canady et al. (2001)
S. habrochaites	Grey mould	*LYC4*	*Rbcq1 and Rbcq2*	Finkers et al. (2007a, b)
	Tomato mosaic virus (ToMV)	–	*Tm-1*	Lévesque et al. (1990); Tanksley et al (1992); Ohmori et al. (1996)
	Tomato yellow curl virus (TYLCV)		*Ty-2*	Hanson et al. (2006)
	Powdery mildew	*GI.1560*	*Ol-1*	Huang et al. (2000a); Bai et al. (2005)
		GI.1290	*Ol-3*	Huang et al. (2000b); Bai et al. (2005)
		PI 247087	*Ol-5*	Bai et al. (2005)
	Leaf mould	PI 370085	Cf-4	Jones et al. (1993); Rivas and Thomas (2005)
S. lycopersicum cerasiforme	Leaf mould	PI 187002	*Cf-5*	Jones et al. (1993); Dickinson et al. (1993); Rivas and Thomas (2005)
	Powdery mildew	LA2172	*ol-2*	De Giovanni et al. (2004); Bai et al. (2008)

Table 15.2 Quantitative resistant loci (QRLs) for various diseases identified in wild species of tomato using molecular markers

Source of resistance	Diseases	Number of QRLs	Mapping populations	Marker type	References
S. arcanum LA 2157	Bacterial canker	3	BC_1	RFLP	Sandbrink et al. (1995)
	Bacterial canker	5	F_2	RFLP, SCAR	Van Heusden et al. (1999)
	Early blight	6	F_2 F_3	AFLP, SSR, SNP	Chaerani et al. (2007)
S. cheesmaniae LA0422	Black mould	5 Bm-2a, 2c, 3, 9, 12	RILs, F6:7	RFLP, CAPS	Cassol and St Clair (1994); Robert et al. (2001)
S. chilense LA1932, LA2779, LA1938	Tomato mottle virus	3	F_2	RAPD	Griffiths and Scott (2001); Ji and Scott (2005)
S. habrochaites LA04O7	Bacterial canker	Rcm 2.0, Rcm 5.1	F_2	RFLP, CAPS	Kabelka et al. (2002); Coaker and Francis (2004)
S. habrochaites PI 126445	Early blight	7–13	BC_1	RFLP, RGA	Foolad et al. (2002a, b)
S. habrochaites PI 126445	Early blight	6 EBR-R9, R26, R27, S2, S15, S70	BC_1, BC_1 S_1	RFLP, RGA	Zhang et al. (2003)
S. habrochaites LYC4	Grey mould	10 Rbcq 1, 2, 3, 4a, 4b, 6, 9a, 9b, 11, 12	ILs, F_2	AFLP, CAPS	Finkers et al. (2007a, b)
S. habrochaites LYC4	Grey mould	3 Rbcq1, Rbcq2, Rbcq4a	ILs, F_2	AFLP, CAPS, SCAR	Finkers et al. (2007a, b)
S. habrochaites LA2099	Late blight	lb4, lb5b and lb11b	BC_1 NIL, Sub NILs	RFLP	Brouwer and St. Clair (2004)
S. habrochaites LA1777	Late blight	5 Rlbq4a, Rlbq4a, Rlbq4b, Rlbq7, Rlbq8, Rlbq12	ILs	RFLP	Li et al. (2011)
S. lycopersicoides	Grey mould	5 Fbc1, 2,3,5,11	ILs	RELP, CAPS	Davis et al. (2009)
S. neorickii G1.1601	Grey mould	3 pQTL3, Pqtl4, pQTL9	F_3, BC_3 S_1, BC_3 S_2	AFLP, RELP, CAPS	Finkers et al. (2008)
S. neorickii G1.1601	Powdery mildew	3 Ol-qtl-1, qtl2, qtl3	F_2, F_3	AFLP, CAPS, SCAR	Bai et al. (2003)
S. neorickii G1.1601	Powdery mildew	2 Ol-qtl1, Ol-qtl2	F_2, BC_2 S_1, BC_2 S_2,	AFLP, CAPS	Faino et al. (2012)
S. peruvianum	Tomato yellow leaf curl	5 TY-5 major+4 minor QRLs	F_3	_	Anbinder et al. (2009)

A recessive gene *ol-2* has also been reported to control resistance to powdery mildew in tomato and associated with papilla formation (Bai et al. 2008). The *ol-2* gene is found in *S. lycopersicum* var. *cerasiforme* (Bai et al. 2008).

In another study, three quantitative resistant loci (QRLs) were identified in the wild species *S. neorickii* G1.1601 (Bai et al. 2003), and these were found to be associated with both HR and papilla formation (Li et al. 2011). These three QRLs were mapped with *Ol-qtl1* on chromosome 6, in the same region as the *OL-1* locus (found in *S. habrochaites*), which is involved in a hypersensitive resistance response to the pathogen. Other two linked QTLs (*Ol-qtl2*, *Ol-qtl3*) were identified on chromosome 12, near the *Lv* locus conferring resistance to the other powdery mildew specie *L. taurica*. These QTLs have been subsequently fine mapped on short arms of chromosomes 6 (*Ol-qtl2*) and 12 (*Ol-qtl3*).

Leaf Mould

Leaf mould is caused by *Cladosporium fulvum* pathogen. This disease generally occurs in severe form under high humidity conditions in protected culture (Watterson 1986). The *Cf-1* was reported to be the first alien gene conferring resistance to this disease (Langford 1937). Since then a total of 21 *Cf* genes have been reported (Haanstra 2000). Wild species *S. habrochaites* has been shown to be an important source of resistance gene *Cf-4*, while resistance genes *Cf-2* and *Cf-9* were identified in the background of *S. pimpinellifolium*. The resistance genes *Cf-4* and *Cf-9* have been transferred to cultivated tomato for development of resistance cultivars.

Using the advanced molecular tools and techniques, many alien genes controlling resistance to tomato leaf mould such as *Cf-2*, *Cf-4* and *Cf-9* have been mapped and cloned through positional cloning, comparative mapping and transposon tagging (Jones et al. 1994; Dixon et al. 1996; Thomas et al. 1997). The gene was identified in PI 187002 accession of *S. lycopersicum cerasiforme* and was mapped to a complex locus on chromosome 6 very closely linked to *Cf-2* (Jones et al. 1993). The isolation of *Cf-5* gene and characterisation of the complex locus was from three genotypes (Dixon et al. 1998). Functional analysis of *S. pimpinellifolium* accessions allowed the identification of novel *Cf* genes (*Cf-ECP1*, *Cf-ECP2*, *Cf-ECP3*, *Cf-ECP4* and *Cf-ECP5*), which trigger horizontal resistance to the leaf mould (Lauge et al. 1998, 2000). The genetic mapping of a new complex locus *Orion* (*OR*) comprises of *Cf-ECP2* and *Cf-ECP3* genes at chromosome 1 (Haanstra et al. 1999; Yuan et al. 2002). Soumpourou et al. (2007) showed that both *Cf-ECP1* and *Cf-ECP4* are located at MW complex locus along with *Cf-4* and *Cf-9* genes.

Grey Mould

Botrytis cinerea fungus infects various fruit, flower and vegetable crops in the field and even after harvest. For greenhouse production specifically stem resistance is a critical trait because pruning provides sites for fungal penetration. Quantitative trait

loci (QTLs) having resistance against *B. cinerea* disease have been identified in the wild species *S. habrochaites* and *S. neorickii* (Finkers et al. 2007a, b, 2008). Ten quantitative trait loci (QTLs) have been identified in the accession LYC4 of *S. habrochaites* illustrating the genetic complexity of resistance to *B. cinerea* (Finkers et al. 2007a, b). Three putative QTLs identified in *S. neorickii* G1.1601 showed high level of resistance (Finkers et al. 2007a, b). Similarly, five putative quantitative trait loci (QTLs) were identified in *Solanum lycopersicoides* LA2951, for resistance in different chromosomes.

Fusarium Wilt

Tomato fusarium wilt is caused by the fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*). Wild species of *Lycopersicon* provide a source of resistance to *Fol*, and dominant resistance genes for this trait have been identified. In tomato, R-genes against the wilt-inducing *Fol* are called *I* (for immunity) genes. Three genes including *I*, *I-2* and *I-3* have been introgressed into commercial cultivars (Huang and Lindhout 1997). The first gene *I* conferred vertical resistance to *race 1* of this pathogen, and it was found in the accession PI 79532 of *S. pimpinellifolium*, assigned on chromosome 11 (Paddock 1950). The gene *I-2* conferring resistance to *race 2* has been discovered in the accession PI 12915 of the same species (Stall and Walter 1965; Cirulli and Alexander 1966). Positional cloning of *I-2* gene showed it as a complex locus. The *I-3* gene (conferring resistance to race 3) was introgressed from *S. pennellii* and is located on chromosome 7 (Bournival et al. 1989). It has been identified in two accessions PI414773 (McGrath et al. 1987) and LA716 (Scott and Jones 1989) of this species and mapped using molecular markers (Tanksley and Costello 1991). The introgression lines have been developed by using the above wild species, and few introgression lines (IL7-2, IL7-3 and IL7-4) showed complete resistance for *Fol* race 3 (Sela-Buurlage et al. 2001; Hemming et al. 2004). Three novel alien genes *I-4*, *I-5* and *I-6* have been identified on different chromosomes of tomato. The loci *I-5* and *I-6* represented new *S. pennellii* resistance loci with varying degrees of potency, while the origin of the *I-4* was not defined. These studies emphasised the complexity of wilt disease resistance at both inter- and intra-locus levels.

Late Blight

The late blight disease in tomato is caused by *Phytophthora infestans*. The wild species *S. pimpinellifolium* was identified as an important source for resistance genes against this disease. Two alien genes (*Ph-1* and *Ph-2*) controlling resistance to this disease have been found in the background of different accessions (Bonde and Murphy 1952; Gallegly and Marvel 1955; Peirce 1971). The gene *Ph-2* had incompletely dominant resistance and provided only a reduction in the rate of disease development rather than blocking the disease. This gene has been mapped on the

long arm of chromosome 10 using molecular markers (Moreau et al. 1998). Later on, gene *Ph-3* was identified in *S. pimpinellifolium* accession L3708 (Chunwongse et al. 2002). This partially dominant gene conferred resistance to a wide range of *P. infestans* isolates showing virulence against the genes *Ph-1* and *Ph-2*. However, since these race specific genes have not been identified as durable against the newly evolved races of *P. infestans*, it has been suggested to combine or pyramid several genes for more durable resistance (Foolad et al. 2008).

Several QTLs conferring race-nonspecific resistance have been reported in tomato wild species *S. habrochaites* (Robert et al. 2001; Brouwer et al. 2004), *S. pennellii* and *S. pimpinellifolium* (Frary et al. 1998). The QTL identified in the accession LA2099 of *S. habrochaites* (Brouwer et al. 2004) and three QTLs have been fine mapped using NILs. These QTLs have been subsequently introduced into tomato breeding lines (Brouwer and St. Clair 2004). However, each NIL carrying one resistance QTLs could not be useful in breeding due to severe linkage drag (Brouwer and St. Clair 2004). Further inspection of the NILs determined that they also contained genes/QTLs for other characteristics such as plant shape, canopy density, maturity, fruit yield or fruit size in the same introgressed regions.

An alien QTLs contributing to LB resistance has also been identified in the accession LA716 of *S. pennellii*. This QTLs has been further validated among the introgression lines derived from cross made between *S. lycopersicum*×*S. pennellii* (Smart et al. 2007). *S. habrochaites* is believed to be a potential donor for high levels of quantitative resistance in tomato (Brouwer et al. 2004). One of the accessions LA1777 of this species showed a good level of resistance against several isolates of *P. infestans* (Li et al. 2011). Five to six consistent QTLs have been identified in another accession LA2099 of *S. habrochaites*, and those lines possessed four major QTLs associated with resistance to *P. infestans* in different environments. Five alien QTLs have also been identified in the accession LA1777 of this species (*Rlbq4a*, *Rlbq4b*, *Rlbq7*, *Rlbq8* and *Rlbq12*). These QTLs showed unambiguous higher levels of resistance, and all these except the QTL *Rlbq4b* have been co-localised with previously described QTLs from *S. habrochaites* LA2099. These studies showed the development of resistant genotypes by combining several QTLs or QRLs, and single genes from one or more wild might pave the way for durable resistance to *P. infestans*.

Early Blight (EB)

The wild species *S. habrochaites* and *S. pimpinellifolium* have been identified as the source of resistance for early blight in tomato. These species have been used to introgress alien genes controlling early blight using traditional backcross breeding. As a result, it led to development of several germplasms with improved resistance to early blight (Table 15.3). However, use of these wild species, particularly *S. habrochaites* in breeding programmes, remains restricted due to linkage drag for undesirable traits such as late maturity, indeterminate growth habit and relatively low-yielding ability.

Table 15.3 Breeding lines developed for late blight resistance in tomato using wild species

Species	Accession used	Breeding line developed	Reference
S. habrochaites	PI 126445	NC EBR-1	Gardner (1988)
	PI 1390662	87B187	Maiero et al. (1990a)
	B 6013	H-7, H-22, H-25	Kalloo and Banerjee (1993)
	Unknown accessions	HRC90.303, HRC 91.279, HRC 91.341	Poysa and Tu (1996)
	PI 126445	NC39E	Foolad et al. (2002a)
S. pimpinellifolium	A 1921	P-1	Kalloo and Banerjee (1993)

Genetic studies on inheritance of alien genes for resistance to this disease revealed that resistance to early blight is under control of many genes (i.e. polygenic) (Çalıs and Topkaya 2011). Therefore, interspecific populations have been developed between cultivated and wild species of tomato (*S. habrochaites*/*S. pimpinellifolium*) in order to map QTLs controlling resistance to EB using molecular markers. Foolad and Sharma (2005) identified 7 QTLs for EB resistance in the background of the accession PI126445 of wild species *S. habrochaites*. This accession has been identified as an important source of resistance (Nash and Gardner 1988) and was involved directly or indirectly in development of improved F_1 hybrids with resistance to early blight such as "Mount Magic". Another wild species *S. arcanum* showed resistance to Indonesian isolate of *A. solani*, and its accession LA2157 has been identified as a strong source of resistance to this isolate.

Bacterial Speck

Bacterial speck disease is predominant in the world and caused by strains of race 0 of *Pseudomonas syringae* pv. *tomato*. The screening of tomato genotypes under field conditions resulted in identification of highly resistance breeding lines (Ontario 7710, Ontario 7611 and Ontario 782) to bacterial speck. Further studies conducted in the laboratory (Pitblado and Kerr 1980) resulted in identification of a single dominant gene controlling the resistance to this disease which was effective against all 98 isolates tested from ten countries. However, subsequent studies showed that *Pto* resistant gene is semidominant or incompletely dominant in nature (Pitblado and MacNeill 1983; Kozik 2002).

Breeding line "Ontario 7710" was used in at least two commercial breeding projects before its identification as a resistance source to bacterial speck due to the presence of many other desirable commercial characteristics. However, subsequently it was used throughout the world as a source of *Pto*. Its complex ancestry showed that gene *Pto* came from a resistant cultivar from Farthest North which had been derived from "Bison" × an accession of *S. pimpinellifolium* (probably PI 370093) (Pitblado and Kerr 1980; Kerr and Cook 1983; Pitblado et al. 1984; Martin et al. 1991). Due to its semidominant nature, this disease is occasionally observed on

commercial hybrids carrying only one copy of *Pto* (Buonaurio et al. 1996). This gene has tight linkage with *Fen*, a gene controlling sensitivity to fenthion, and hence can be used as marker for selecting *Pto* gene. For obtaining complete resistance to race 0 of *P. syringae* pv. tomato in hybrid varieties, it is required to introgress the both *Pto* and *Prf* genes. Both these genes have been cloned (Loh and Martin 1995; Salmeron et al. 1996), and available gene sequences can be used to develop gene-based markers for pyramiding these genes through marker-assisted selection.

Bacterial Spot

Tomato bacterial spot is caused by *Xanthomonas* spp. Four different species and at least five races of these species have been recognised as causal agents including *X. euvesicatoria* (race T1), *X. vesicatoria* (race T2), *X. perforans* (race T3, T4, T5) and *X. gardneri* (race T2) (Jones et al. 2004, 2005). The alien source for resistance against this disease has been identified in the accession LA716 of wild species *S. pennellii*. A dominant resistance locus, *RXopJ4* was recognised in this accession which was previously referred to as *Xv4* and was mapped on chromosome 3 (Astua-Monge et al. 2000).

Bacterial Canker

Bacterial canker of tomato, caused by the gram-positive bacterium *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), is a devastating disease that proliferates in the xylem vessels of infected plants. Two quantitative trait loci (*Rcm 2.0* and *Rcm 5.1*) controlling resistance to this disease have been identified in the background of the accession LA407 of *S. habrochaites*. These loci were introgressed into *S. lycopersicum L.* Mill. genetic background using inbred backcross breeding (Kabelka et al. 2002).

Tomato Yellow Leaf Curl Virus (TYLCV) and Tomato Leaf Curl Virus (ToLCV)

Tomato yellow leaf curl virus (TYLCV) is one of the most devastating viruses of cultivated tomato in tropical and subtropical regions in the world. Although first identified in the eastern Mediterranean, it has now spread worldwide (Barbieri et al. 2010). Genes controlling resistance to TYLCV have been identified in wild species of tomato (e.g. *S. pimpinellifolium*, *S. peruvianum*, *S. chilense* and *S. habrochaites*) (Ji et al. 2007a). Conventionally, use of wild species for introgression of alien resistant genes into cultivated species was initiated in Israel in the late 1960s (Pilowsky and Cohen 1974). As a result, first commercial hybrid (TY20) resistant to TYLCV was released in 1988 (Pilowsky and Cohen 1990). This hybrid variety carried resistance from *S. peruvianum* (accession PI 126935). However, the type of resistance, the

availability of the infected vectors and the presence of different viruses causing TYLCD make classical breeding slow and difficult for this disease because phenotypic analyses of partial resistance or tolerance traits are often of limited utility. Marker-assisted breeding overcomes such problems focusing on the direct selection of genomic loci underlying the trait (Barbieri et al. 2010).

The resistance locus *Ty-1* was identified in the background of accession LA1969 of wild species *S. chilense*. This gene confers partially dominant resistance and has been introgressed into cultivated tomato. As the result, several partially resistant lines such as LA3473 have been developed (Michelson et al. 1994; Zamir et al. 1994). Another gene, *Ty-2*, is derived from B6013 accession of *S. habrochaites* (Hanson et al. 2000), and in India, this source has been used to develop several TYLCV-resistant tomato lines (Kalloo and Banerjee 1990). Among these lines, H24 breeding line showed excellent TYLCV resistance in Taiwan and southern India. These lines were studied further, which resulted in identification of a novel locus and its association with molecular markers (TG105A and T0302) on the long arm of chromosome 11 (Ji et al. 2007a). Another begomovirus resistance locus, *Ty-3*, has been identified on same genomic region that carried *Ty-1*gene (Ji and Scott 2006). Ji et al. (2007b) identified a large *S. chilense* introgression carrying *Ty-3* in LA2779-derived advanced breeding lines resistant to both TYLCV and tomato mottle virus (ToMoV). Molecular markers linked to resistance genes, namely, *Ty-4* and *Ty-5*, have also been identified (Anbinder et al. 2009; Ji et al. 2009a, b). Vidavski et al. (2008) reported a pyramiding strategy, by classical breeding, of different sources of resistance to TYLCV coming from different wild species. The preferential line TY172 had been derived from four different accessions, namely, PI 126930, PI126930, PI 390681 and LA 441 of *S. peruvianum* (Friedmann et al. 1998). TY172 is highly resistant to TYLCV and QTL mapping showed that resistance in TY172 is controlled by a major QTL *Ty-5* and four minor QTLs (Anbinder et al. 2009).

Tomato Spotted Wilt Virus (TSWV)

The disease is caused by a Tospovirus, which transmits naturally by thrips, including the tobacco thrips, *Frankliniella fusca*, and the Western flower thrips, *F. occidentalis*. So far, several genes for TSWV resistance have been reported (*Sw1a*, *Sw1b*, *sw2*, *sw3*, *sw4*, *Sw-5*, *Sw-6* and *Sw-7*) (Finlay 1953; Price et al. 2007; Saidi and Warade 2008). However, most of these genes could not be used in commercial varieties because resistance was quickly overcome (Price et al. 2007; Saidi and Warade 2008) and only alien genes (*Sw1a*, *Sw-5*, *Sw-6*, *Sw-7*) derived from different wild species have been exploited in tomato improvement. Among these genes, *Sw-5* has been most widely used to deploy TSWV resistance gene due to its durability to multiple tospoviruses. It was first identified in wild species *S. peruvianum*. *Sw-6* conferred partial resistance to thrips inoculation and showed a narrower range for resistance to viral isolates than *Sw-5* (Rosello et al. 1998, 2001). Another gene *Sw-7* has also been identified and introgressed from *L. chilense* LA 1938 into *L. esculentum* (Canady et al. 2001; Stevens et al. 2006; Price et al. 2007; Saidi and Warade 2008).

AFLP marker has been identified to be linked to the gene (Price et al. 2007), and the gene was mapped on chromosome 12 flanked by the markers T1263 and SSR20 (Stevens et al. 2009). An accession LA 1938 of another wild species *L. chilense* showed acceptable levels of resistance to TSWV. This new source carries highly resistant single dominant gene *Sw-7*. Presently, the *L. chilense*-based germplasm is being tested in Australia, Thailand and Taiwan and plans to test it in Italy are underway (Stevens et al. 2006).

Cucumber Mosaic Virus

Solanum chilense LA1932 and *S. lycopersicum* backcross introgression lines were screened for tolerance to lethal necrosis induced by CMV-Fny/77-satRNA (phenotype pattern 3); the tolerant phenotype was observed in 33 % of plants of the BC_1F_2 progeny and 1 % of plants of the BC_1F_3 progeny. Thus, potentially useful sources of tolerance to CMV/satRNA-induced diseases were identified, although the tolerant phenotypes appeared to be controlled by complex quantitative trait loci.

15.4.1.2 Insect-Pest Resistance

Tomatoes are susceptible to a wide array of arthropod insect-pests. Currently available cultivars do not have sufficiently high levels of insect-pest resistance to allow for significant reductions in the amount of pesticides used in the crop. Consequently, developing cultivars with increased levels of insect-pest resistance is a major focus of tomato breeding programme, along with their adoption into integrated pest management programme aimed at reducing pesticide sprays and environmental protection (Table 15.4).

Whitefly

Whitefly (*Bemisia tabaci*) can cause serious problems in the cultivation of tomatoes and other vegetable crops, mainly because they are vectors for a large number of harmful viruses. In addition, feeding of whiteflies inhibits plant growth, and the honeydew produced by the whiteflies can promote sooty mould growth, which may lead to physiological disorders. Whitefly resistance has been found in wild relatives of tomato such as *S. pennellii*, *S. habrochaites*, *S. chilense*, *S. pimpinellifolium* and *S. galapagense*. Whitefly resistance in wild relatives of cultivated tomato is suggested to be associated with glandular trichomes. Of the seven trichome types found in tomato and its wild relatives, four types are glandular. Glandular trichomes might play a role as physical barrier and/or source of compounds deterrent and/or toxic to whiteflies, and the presence of type IV and VI trichomes is highly correlated with whitefly resistance.

Table 15.4 Resistant genes/QTLs identified in wild species of tomato using molecular markers

Resistance source and accession	Insect/pathogen	Traits associated with resistance	Number of genes/QTLs	Mapping populations used	Marker type	References
S. galapagense PRI95004	Whitefly	Type IV trichome	*2 Wf-1, Wf-2*	F_2, F_3	SNP	Firdaus et al. (2013)
S. habrochaites LA1777	Whitefly	Glandular trichomes (type IV)	*4 R1/10, R2/9, R3/11a, R4/11b*	F_2	CAPS, PCR	Momotaz et al. (2010)
S. pennellii LA716	Silver leaf	Acylsugars	*5 TA 4,5,6,10.1,11*	BC_1 F_1, BC_1 F_2	CAPS, SSR, PCR	Mutschler et al. (1996); Leckie et al. (2012)
S. pennellii LA716 (breeding line CU071026)	Whitefly	Acylglucoses	*3 AG 3.2, AG4, AG 11*	BC_1 F_1, BC_1 F_2		Leckie et al. (2012)
S. pimpinellifolium TO-937	Two-spotted spider mite	Acylsucrose accumulation and type IV glandular trichome density	*2 Rtu2.1 Rtu2.2*	F_4, F_8	SNP, PCR	Salinas et al. (2012); Alba et al. (2009)
S. peruvianum PI 128657	Nematodes	–	*Mi-1 (Meu1)*	NILs, F_2	RFLP, ISO	Milligan et al. (1998)
S. peruvianum PI 126443-1MH	Nematodes	–	*Mi-3*, *Mi-5*	BC, F_2	RAPD, RFLP	Veremis and Roberts (1996a, b, c)
S. arcanum LA 2157	Nematodes	–	*MI-9* (heat-stable resistance)	F_2, F_3	RFLP, PCR	Ammiraju et al. (2003)
S. pimpinellifolium LA0121	Potato cyst nematode	–	*Hero*	NIL, F_2	RAPD, RFLP	Ganal et al. (1995); Ernst et al. (2002)

Acylsugars are broad-spectrum insect resistance sugar esters produced at very high levels by some accessions of the wild tomato including *Solanum pennellii* LA716 (Shapiro et al. 1994). This accession produces very high levels of these compounds which have been shown to be effective at controlling a wide variety of insect-pests. The acylsugars of *S. pennellii* LA716 are produced as droplets exuded from type IV glandular trichomes found on all aerial parts of the plant except the flowers.

Acylsugars are the major exudates of type IV trichomes in *S. pennellii* and *S. pimpinellifolium* also (Leckie et al. 2012). *S. habrochaites* LA1777 contains volatile compounds, including germacrene D, dedecatriene and a-farnesene, and has demonstrated high levels of repellent and fumigant activity against *B. tabaci* adults. Momotaz et al. 2010 identified the four QTLs in different chromosomes in *S. galapagense* LA PRI95004. Two QTLs, namely, major QTL (*Wf-1*) and a minor QTL (*Wf-2*), were also identified for whitefly resistance providing for adult survival co-localised with type IV trichome characteristics (presence, density, gland longevity and gland size) (Firdaus et al. 2013).

Transferring acylsugar production from *S. pennellii* LA716 to cultivated tomato through traditional breeding developed the benchmark acylsugar breeding line CU071026. The base moiety of acylsugars (sucrose vs. glucose) can vary among *S. pennellii* accessions. In *S. pennellii*, 12 QTLs were detected for presence and density of type IV trichome and production of acylsugar (Leckie et al. 2012). Additionally, the accession *S. pennellii* LA716 produces almost exclusively acylglucoses, but the breeding line CU071026 derived from *S. pennellii* LA716 produces exclusively acylsucroses. Leckie et al. (2012) identified three quantitative trait loci (QTLs) on chromosomes 3, 4 and 11 from *S. pennellii* LA716 acylsucroses breeding line CU071026. Potential sources of resistance to spider mite have been identified within wild-related species of tomato such as *S. pennellii*, *S. habrochaites* and *S. habrochaites glabratum*.

Red Spider Mite

All commercial varieties of tomato are susceptible to two-spotted spider mite, *Tetranychus urticae* Koch, a serious tomato pest in temperate regions. The spider mites suck the contents of the plant cells, causing tiny pale spots or scars where the epidermal cells have been destroyed. Later stages of the infestation entail chlorosis, defoliation and even plant death, which contribute to a significant crop loss. The density of type IV and VI glandular trichomes and the presence of volatile compounds in trichome gland secretions have been described as factors mediating resistance in some accessions of *S. habrochaites* and *S. pennellii* (Alba et al. 2009). A novel source of resistance to two-spotted spider mite was found in *Solanum pimpinellifolium* L. accession TO-937. Two QTLs, namely, *Rtu2* and *Rtu2.2*, have been identified for resistance to this pest (Salinas et al. 2012). The *Rtu2* QTL not only serves as a valuable target for marker-assisted selection of new spider mite-resistant tomato varieties but also as a starting point for a better understanding of the molecular genetic functions underlying the resistance to this pest.

Root-Knot Nematodes

Gene *Mi-1*, introgressed from *L. peruvianum* PI 128657, is the only commercially available source of resistance to root-knot nematodes in tomato. This gene has been exploited extensively in the last two decades for modern tomato cultivar development. *Mi-1* confers resistance to three species of root-knot nematodes, *Meloidogyne arenaria*, *M. incognita* and *M. javanica* (Dropkin 1969a), as well as to the potato aphid (*Macrosiphum euphorbiae*) (Rossi et al. 1998). Genetic and physical mapping localised *Mi-1* in the introgressed region on the short arm of chromosome 6. *Mi-1* was cloned and shown to belong to the class of resistance genes that contain a leucine zipper, nucleotide-binding site and leucine-rich repeats (Milligan et al. 1998). Although *Mi-1* is a very effective source of root-knot nematode resistance in the field, *Mi-1*-mediated resistance is inactive above 28 °C soil temperature (Holtzmann 1965; Dropkin 1969b).

In the *Mi-1* locus on the short arm of tomato chromosome 6, *Mi-1* and six homologues exist in two distinct clusters about 300 kb apart (Vos et al. 1998; Seah et al. 2004). The cluster containing *Mi-1* (also known as *Mi-1.2*) has two additional members, *Mi-1.1* and *Mi-1.3*, and is located near the centromeric proximal end of the chromosome (Kaloshian et al. 1998; Milligan et al. 1998). *Mi-1.3* is a pseudogene, while *Mi-1.1* and *Mi-1* are both transcribed genes with over 91 % sequence identity (Milligan et al. 1998). The additional linked gene *Mi-5* for heat-stable resistance was found in the same region of *Mi-3*. Two weakly linked pair of genes (*Mi-2*, *Mi-8* in PI 270435 clone 2R2 and *Mi-6* and *Mi-7* in PI 270435 clone 3MH) which seemed to be independent of each other and away from the *Mi-3*/*Mi-5* region were found on chromosome 12. The short arm of chromosome 6 is characterised by clusters of disease resistance R-genes besides *Mi-1* and *Mi-9*.

In different accessions of *S. peruvianum*, several heat-stable resistance genes have been characterised and their allelic relationships have been determined (Veremis and Roberts 1996a, b, c). In LA2157, an accession belonging to the ancient Maranon race complex of *S. peruvianum* from the Maranon drainage area located in northern Peru, the heat-stable root-knot nematode resistance is also mediated by a single dominant gene, *Mi-9* (Veremis et al. 1999). This gene confers resistance to *Mi-1*-avirulent isolates of *M. arenaria*, *M. incognita* and *M. javanica* at 25 and 32 °C but does not confer resistance to *Mi-1*-virulent nematodes.

15.4.2 Abiotic Stress

Sources of genetic tolerance (or resistance) to different abiotic stresses are found in several wild species, including *S. pimpinellifolium*, *S. peruvianum*, *S. cheesmaniae*, *S. habrochaites*, *S. chmielewskii* and *S. pennellii*. Alien genes/QTLs controlling different stresses in these species have been identified and may be utilised in tomato breeding for stress tolerance through marker-assisted selection (Table 15.5).

Table 15.5 QTLs for tolerance/resistance to abiotic stresses identified in wild species of tomato

Tolerance/resistance source	Abiotic stress	Number of QTLs	Mapping populations	Marker type	References
S. pimpinellifolium LA0722	Cold tolerance	3	BC_1 S_1	RFLP	Foolad et al. (1998)
S. habrochaites		3	BC_1	ISO	Vallejos and Tanksley (1983)
S. habrochaites LA1788		10	BC_1	RFLP	Truco et al. (2000)
S. pimpinellifolium LA0722	Drought tolerance	4	BC_1 S_1	RFLP	Foolad et al. 2003
S. pennellii LA0716		3	BC_1 S_1, F_3	RFLP	Martin et al. (1989)
S. pennellii LA0716	Salt tolerance	5	F_2	ISO	Foolad and Jones (1993)
S. pennellii LA0716		8	F_2	RFLP, ISO	Foolad and Lin (1997)
S. pennellii LA0716		8	F_2	RAPD	Foolad and Chen (1998)
S. pimpinellifolium LA0722		7	BC_1 S_1	RFLP	Foolad et al. (1998)
S. pennellii LA716		4	IL (IL6-2, IL7-1, IL7-5)	RFLP	Li et al. (2011)
S. lycopersicoides LA2951		6	IL	RFLP	Li et al. (2011)
S. pennellii LA0716		6	F_2	ISO	Zamir and Tal (1987)
S. pennellii LA716		125	IL	RFLP, PCR	Frary et al. (2010)
S. pimpinellifolium LA0722		4	BC_1 S_1	RFLP	Foolad and Chen (1999)
S. pimpinellifolium LA0722		5	BC_1	RFLP	Foolad et al. (2001)
Solanum lycopersicum var. *cerasiforme* × *S. pimpinellifolium*, *S. galapagenese*		18,25	Two F_8 RILs	RFLP, SSR, CG	Villata et al. (2008)
S. galapagenese (L2) × *S. pimpinellifolium* (L1 and l5)		31	Three F_2	RFLP, ISO	Monforte et al. (1997a)
S. galapagenese (L2) × *S. pimpinellifolium* (L1 and l5)		45	Three F_2	RFLP, ISO	Monforte et al. (1997b)
S. pimpinellifolium L1		6	F_2, F_3	ISO, RAPD, RFLP	Breto et al. (1994); Monforte et al. (1996)
S. pimpinellifolium L1		12	F_2	ISO, RFLP	Monforte et al. (1996)
S. galapagenese L2		8	F_2	RFLP, ISO	Monforte et al. (1999)
Solanum lycopersicum var. *cerasiforme* × *S. pimpinellifolium*, *S. galapagenese*		12, 23	Two F_7 RILs	RFLP, SSR, CG	Villalta et al. (2007)
L. pennellii LA716	NaCl tolerance	>20	IL	RFLP	Frary et al. (2011)

15.4.2.1 Cold Tolerance (CT)

The cultivated tomato is highly sensitive to low temperatures: growth and development is inhibited under 10 °C, and temperatures below 6 °C can cause irreparable damage. The physiology and genetics of tomato cold tolerance (CT) has been investigated at different developmental stages. The cold tolerance was studied in the interspecific population derived from crossing between *S. lycopersicum* and *S. pimpinellifolium*. The genetic basis for shoot wilting and root ammonium uptake under chilling temperatures was examined in an interspecific backcross (BC_1) population derived from *S. lycopersicum* cv T5 and *S. habrochaites* LA1788 which showed that there are around ten QTLs responsible for cold tolerance (Truco et al. 2000). However, QTL mapping studies for cold tolerance during vegetative growth and reproduction are scarce. This is unfortunate, as the value of QTLs for cold tolerance at these stages would be much greater than that during seed germination since most field tomato productions are based on the use of transplants that are often produced in warm greenhouses. Nonetheless, tomatoes with cold tolerance during seedling stage can facilitate early field planting, which may lead to early harvest and huge economic incentives.

15.4.2.2 Drought Tolerance (DT)

Drought, defined as the occurrence of a substantial water deficit in the soil or atmosphere, is an increasingly important constraint to crop productivity and yield stability worldwide. It is by far the leading environmental stress in agriculture, and the worldwide losses in yield owing to this stress probably exceed the losses from all other causes combined. Development of drought tolerance genotypes is still a major challenge to plant breeders, partly because of the perceived complexity of this trait. In tomato, limited studies have been conducted to identify the genes/QTLs in the wild species, and only one study reported identification of four QTLs in backcross progeny of a *S. lycopersicum* × *S. pimpinellifolium* cross (Foolad et al. 2003). The stability of these QTLs across other populations and interspecific crosses, however, should be examined before their introgression into the cultivated tomato through MAS.

15.4.2.3 Salt Tolerance (ST)

Salt tolerance can be defined as the ability of plant to survive and maintain growth under saline conditions. A better approach for improving the efficiency of selection for complex traits is to discover genetic markers associated with genes or QTLs controlling the traits imparting salt tolerance. Molecular markers also facilitate pyramiding of individual tolerance components from different sources. Few accessions of wild species (*S. pimpinellifolium*, *S. peruvianum*, *S. cheesmaniae*, *S. habrochaites*,

S. chmielewskii and *S. pennellii*) adapted to dry or seashore regions have shown certain levels of salt tolerance (Foolad and Lin 1997). The genomic regions controlling salt tolerance during seed germination, vegetative growth and later stages in tomato have been identified in interspecific populations. The comparative study of QTLs identified for salt tolerance in different interspecific populations of tomato such as *S. lycopersicum*×*S. pennellii* and *S. lycopersicum*×*S. pimpinellifolium* crosses indicated conservation of some QTLs across species. Several studies have identified stable QTLs for salt tolerance in the background of wild species including *S. pennellii*, *S. lycopersicoides*, *S. galapagenese* and *S. pimpinellifolium* (Breto et al. 1994; Foolad 2004; Monforte et al. 1996, 1997a, b, 1999; Villalta et al. 2007; Li et al. 2011). Five QTLs for salt tolerance have also been identified in an interspecific population derived from between *S. lycopersicum* and *S. pimpinellifolium* LA0722 (Foolad et al. 2001).

15.5 Development of Introgression Lines (ILs) from Wild Species

Many agronomically important traits as well as disease resistance have quantitative inheritance in tomato. These traits are controlled by a combined action of QTLs with favourable alleles often present in the wild species (Fulton et al. 2000; Bai et al. 2003). These favourable QTLs can be introgressed into cultivated background on the basis of marker-trait association knowledge by designing optimal breeding strategies. In tomato, interspecific populations have been extensively used for mapping such QTLs. However, in an interspecific cross, multiple segregating QTLs at the whole genome level often tend to mask the effects of one another (Tanksley and Nelson 1996; Grandillo et al. 1999). On the other hand, introgression lines (ILs) are more often identified as powerful resources for identification of such QTLs because these lines carry only a single introgressed region, while the rest of their genome is identical. As a result, the phenotypic variation in these lines can be associated with individual introgression segments. In tomato, several sets of ILs carrying alien genes from wild relatives have been developed using *S. pennellii* (Eshed and Zamir 1996) and *S. habrochaites* (Monforte and Tanksley 2000) species. Introgressions have also been made with *S. lycopersicoides* and *S. sitiens* (Chetelat and Meglic 2000; Canady et al. 2006). These genomic resources are available in public domain upon request from the Tomato Genetics Resource Center in Davis, California (http://tgrc.ucdavis.edu). Introgression lines carrying genes/QTLs for quantitative traits are useful for utilising in breeding programme, and different genomic regions of wild tomato species can be pyramided into new breeding lines (Fridman et al. 2004). Thus, ILs (also called prebreds) will offer tomato breeders a powerful tool to optimise the uses of genetic variation in nature by bringing together in one genotype alleles that maximise yield, resistance to biotic and abiotic stress, etc.

15.6 Development of Improved Cultivars Using Alien Genes

Beside natural introgressions, wild species have also been extensively used as important sources of resistance genes against various diseases, insect-pests and abiotic stresses. Inheritance and mapping of these alien genes/QTLs have been done using molecular markers as well as traditional breeding methods. As a result, the development of improved cultivars having not only resistance to various diseases/pests but also higher yield has been revolutionised. Alien gene introgression also led to development of prebreeding lines which have been subsequently used as donors for development of improved high-yielding resistant cultivars in several countries. In USA, a number of tomato cultivars including F_1 hybrids have been developed using alien genes as resistant sources either directly or indirectly. "Pan American" was the first commercially released cultivar that was developed from direct introgression of alien genes from wild species (through the cross Marglobe × *S. pimpinellifolium*) (Porte and Walker 1941). Earlier, *Fusarium* wilt-resistant variety "Marvel" (Gardner 1920), which was a selection from Merveille des Marchés in the early 1900s, was used indirectly as a resistant source in development of tomato variety "Marglobe" released in 1925. Subsequently "Marvel" was a parent of many important varieties from the 1930s through the late 1950s (Labate and Robertson 2012). Globally, breeding efforts to introgress the alien genes were increased after World War II (Rick and Chetelat 1995), and these are still being continued, albeit using sophisticated tools today such as introgression libraries for gene discovery (Zamir 2001, 2008).

Use of molecular markers in backcrossing could be very useful to eliminate genetic linkage of nontargeted loci. Several examples of linkage drag in tomato and other crops have been quantified using molecular markers (Haanstra et al. 1999; van der Beek et al. 1992; Young et al. 1988; Randhawa et al. 2009). Two breeding lines 071733-1 and 071733-4 having alien genes resistant to early blight and late blight have been developed by crossing two wild species (*S. pimpinellifolium* and *S. hirsutum*). These lines also had strong SLS resistance which was shown to be governed by a single gene. These lines have been used to develop "triple resistant" lines having alien resistance genes for SLS, EB and LB. The molecular markers helped to identify the homozygous lines possessing genes for late blight (*Ph3* and *Ph2*), early blight and Septoria leaf spot. These "triple resistant" lines with good fruit size have been used to rapidly transfer these resistances to their newest tomato varieties (Bornt and Zitter 2012). Recently, a F_1 hybrid variety "Mountain Magic" having resistance to early and late blight was released for cultivation. One of the parents (NC 2 CELBR) involved in development of this variety has been derived from two wild species *L. pimpinellifolium* and *L. hirsutum* having resistant genes for early and late bight (Gardner and Panthee 2012). A study showed very good resistance of foliar symptoms of late blight in all tomato varieties and experimental hybrids having alien genes *Ph2* and/or *Ph3*. These varieties, most developed in the USA, are Plum Regal (*Ph3*), JTO-545 (*Ph3*), Legend OP (*Ph2*), Matt's Wild Cherry (undetermined resistance, possibly *Ph3*), Jasper (undetermined resistance, likely *Ph2* and/or *Ph3*) and Defiant PHR, Mountain Magic and Mountain Merit. Iron Lady is a new variety having both *Ph2* and *Ph3* and expected to have even better resistance than the others

species (Anonymous 2013). The other varieties such Mountain Fresh, Mountain Crest, Mountain Pride, Walter, Sun leaper and Mountain Merit have been reported resistant to late blight, early blight, wilt and TSWV from wild species. These resistant varieties to various diseases of tomato have been developed from those parental lines that carried the resistance genes from the wild background.

15.7 Conclusion

Generally, the use of wild relatives for the improvement of complex traits important to agriculture, including yield, quality and tolerance to biotic and abiotic stresses, has been limited. There are several problems, in fact, associated with the utilisation of wild species, which have in many cases deterred breeders from using them. These include the pre- and post mating barriers, the presence of several undesirable loci that might be transferred along with the traits of interest (linkage drag), the complexity and the time necessary to recover the elite genetic background while selecting for the desired characters and the inferior phenotype of the wild germplasm for many of the traits that breeders would like to improve. From the present chapter, it is evident that wild species have been utilised the worldover to address biotic and abiotic problems, specifically, by resolving various constraints which had been slowing the process of introgression of genes of interest. Tremendous progress has however been made in the development of improved innovative germplasm for further utilisation in hybridisation programme across the biotic and abiotic stress problems. Significant achievements with respect to varietal development had been mainly in case of tomato leaf curl virus biotic stress. In this case, open-pollinated varieties/hybrids developed by introgressing genes for resistance from wild species have been able to put rainy season tomato cultivation in tropical and subtropical areas back on track vis-a-vis saving the environment and consumers from pesticide pollution. It would, therefore, be important to continue to access the wild relatives and unadapted sources of the crop plants so that new alleles could be available for future improvement. Further, the information available from tomato genome sequencing is expected to improve the efficiency with which wild tomato relatives will contribute to improvement of this important crop.

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Chapter 16
Eggplant

Giuseppe Leonardo Rotino, Tea Sala, and Laura Toppino

Abstract The eggplant is one of the most important solanaceous crops and is widely cultivated across the world for its fruits, mainly used as a vegetable. Wild relatives of eggplant have been shown to be important sources for transferring tolerance to biotic and abiotic stresses and other agronomically important traits in cultivated background. However, most of the wild relatives have shown partial cross-compatibility with the cultivated species. Efforts have been made to transfer alien genes controlling important traits into the cultivated gene pool of eggplant (*S. melongena*) using the sexual, somatic hybridisation and genetic transformation methods. Introgression lines and molecular markers have accelerated the identification of alien segments introgressed from wild species and helped assessing their impact on phenotypic expression. These approaches have opened new ways to solve constraints for accessing to the reservoir of alien alleles represented by the progenitors, allied and wild species of *S. melongena*. This chapter focuses on such developments and the major achievements made through alien gene transfer in eggplant.

Keywords Dihaploids • Genetic engineering • Genetic resources • Linkage drag • QTL • *S. melongena* • Somatic hybridisation

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16.1 Introduction

The eggplant, *Solanum melongena* L. ($2n = 24$), also known as brinjal, belongs to the *Solanaceae* family, and similar to the other popular and important solanaceous crop such as tomato, potato and pepper, is cultivated across all continents. Eggplant reached in 2010 a harvested area of 1,674,092 ha with a production of 43,891,773 tons of berries showing an increase, respectively, of 9 % and 60 % when compared to those of 2000 (FAOSTAT, http://faostat.org, 2012). This crop is especially cultivated in Asia (93 % of both the world production and harvested area) particularly in China, India, Indonesia and Iran followed by Africa and Europe with, respectively, about 3.3 % and 2.3 % of harvested area and 3.6 % and 2.1 % of world production, being Egypt and Italy the most interested countries. This crop needs warm weather for an extended period of time to give good yields; therefore the tropical and subtropical climates are considered optimal for its cultivation; however it is also grown in temperate and continental regions both in an open field and under protected cultivation for off-season production. Although eggplant is generally considered as a "low-calorie vegetable", its nutritional value is comparable to most of the other common vegetables. It contains vitamins, minerals, proteins, fibre and also important phytonutrients like phenolic compounds and flavonoids, many of which have antioxidant activity (Raigona et al. 2008). Besides, eggplant has been used in the traditional medicine. The tissue extracts are used for the treatment of asthma, bronchitis, cholera and dysuria; fruits and leaves are beneficial in lowering blood cholesterol. It has been shown that eggplant also possesses antimutagenic properties (Khan 1979; Hinata 1986; Kalloo 1993; Collonnier et al. 2001a; Kashyap et al. 2003; Rajam and Kumar 2006). As other solanaceous crops, eggplant contains the glycoalkaloids solasonine and solamargine, which may exert potential toxic effect on human beings but have also shown anticancer properties; in particular, skin neoplasias are efficiently treated with commercial cream based on eggplant-derived glycoalkaloids (Cham 2012).

The cultivated eggplant exhibits a wide diversity in phenotypic (e.g. colour, shape and size of plants, leaves and fruits; growth habit; prickliness and hairiness), physiological and biochemical characteristics, and it is susceptible to a variety of biotic and abiotic stresses, which may cause great yields and quality losses. The classical breeding methods, by exploiting mainly the intraspecific genetic diversity, have accomplished the development of several varieties of eggplant improved with regard to the fruit size, weight and shape and to the plant resistance to diseases and pests (Kalloo 1993). Moreover, interspecific crosses of eggplant with its wild and allied relatives have been attempted together with biotechnological approaches, such as somaclonal variation, somatic hybridisation and genetic transformation, to further enlarge the genetic variability for improving the resistance to pests and diseases, the quality and, more recently, the nutritional value of the fruit. This chapter reviews the progress made for introgression of alien genes into the cultivated eggplant and their potential use in breeding programmes.

16.2 Wild Genetic Resources

In Europe and some Asian countries, and particularly where the cultivation system became more intensive, the release of F_1 hybrids has contributed to the loss of eggplant landraces thus, unavoidably, causing genetic erosion in *S. melongena* (Daunay et al. 1997). Therefore, it is greatly needed, through conventional breeding methods and biotechnological approaches, to introgress the traits of resistance to diseases and pests from the *S. melongena* progenitors, landraces, allied and wild relatives into the gene pool of cultivated eggplant. Germplasm resources are an important reserve of potential genetic variability and allelic variation for the traits underlying many agronomic and qualitative features of the plant and fruit as well as for the content of nutritional and functional compounds. Since 1977, actions aimed at preserving the genetic resources of the cultivated eggplants (including *S. melongena*, *S. aethiopicum* and *S. macrocarpon*) and its relative have been undertaken in Europe, Asia and Africa (Lester et al. 1990; Collonnier et al. 2001a). In the case of eggplants, the controversial identification of its progenitors and of its centres of origin and domestication (Weese and Bohs 2010; Meyer et al. 2012) has also been a hindrance to the search for useful genetic variability with regard to the germplasm and to the geographical areas. In fact, taking into account the enormous contribution that the use of wild relatives and progenitors have given to the genetic improvement of the solanaceous species tomato and pepper (Lenne and Wood 1991), it emerges that for eggplant the potential usefulness of its allied and wild relatives is far to be fully exploited. The allied species *S. aethiopicum* gr. *gilo* and gr. *aculeatum* (Fig. 16.1a), *S. incanum* and *S. macrocarpon* have been frequently subjected to evaluation and utilised as source of genetic variability because they are considered genetically closer to *S. melongena*.

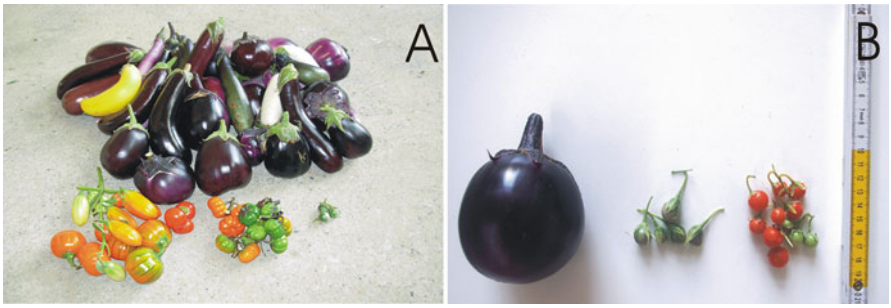

Fig. 16.1 (**a**) Fruits of different introgression lines of *Solanum melongena* and of three allied species utilised for somatic and sexual hybridisation (top of the image, eggplant introgression lines; bottom, from the left, *S. aethiopicum* group *gilo*, *S. aethiopicum* group *aculeatum* = *S. integrifolium* and *S. sodomaeum*) and (**b**) sexual hybrids between eggplant and *S. tomentosum*, from left: fruit of eggplant line from purpura typology, of the sexual F_1 hybrid with *S. tomentosum* and of *S. tomentosum*

16.3 Important Traits in Wild Relatives

Eggplant is susceptible to numerous diseases and pests. Particularly, soilborne diseases (bacterial and fungal wilts, nematodes) and insects are the most serious cause of yield losses both in greenhouse and in open-field cultivations (Sihachakr et al. 1994). Partial resistance/tolerance to most pathogens is also found within the eggplant gene pool, but its low efficacy, often, hindered an effective employment in breeding programmes (Daunay et al. 1991). Resistance to bacterial wilt (*Ralstonia solanacearum*) in some eggplant varieties has become ineffective during hot planting seasons or in badly drained fields (Ano et al. 1991). Only partial resistance or weak tolerance against root-knot nematodes (*Meloidogyne* spp.), *Verticillium* (*Verticillium dahliae*) and *Fusarium* (*Fusarium oxysporum* f. sp. *melongenae*) wilts, *Phomopsis* blight (*P. vexans*) and the insects *Leucinodes orbonalis* (shoot and fruit borer), *Amrasca biguttula* (leaf hopper) and *Aphis gossypii* has been reported in some varieties of eggplant (Yamakawa and Mochizuki 1979; Bindra and Mahal 1981; Chelliah and Srinivasan 1983; Sambandam and Chelliah 1983; Messiaen 1989; Ali et al. 1992; Rotino et al. 1997a). Table 16.1 shows the wild and allied species of eggplant which have been reported to carry traits of resistance to most diseases and pests affecting eggplant. It can be noted that *S. sisymbrifolium* and *S. torvum* have been recognised as resistant to the most severe diseases of eggplant, such as the soilborne

Table 16.1 Sources of resistance against diseases and pests in wild and allied species of eggplant

Pathogens/pests	Resistant species	References
Fungi		
Leveillula taurica	*S. laciniatum*, *S. nigrum*, *S. linneanum, S. aculeatissimum*, *S. aviculare*, *S. pseudocapsicum*	Bubici and Cirulli (2008)
Phomopsis vexans	*S. viarum*, *S. sisymbrifolium*, *S. aethiopicum* gr gilo, *S. nigrum*, *S. violaceum*, *S. incanum*	Kalda et al. (1977); Rao (1981)
Fusarium oxysporum	*S. violaceum* Ort., *S. incanum* agg. *S. mammosum* L., *S. aethiopicum* gr *aculeatum* and gr *gilo*, *S. torvum*	Yamakawa and Mochizuki (1979); Rizza et al. (2002)
Fusarium solani	*S. aethiopicum* L. gr *aculeatum*, *S. torvum* SW.	Daunay et al. (1991)
Verticillium dahliae and *V. albo-atrum*	*S. sisymbrifolium*, *S. aculeatissimum*, *S. Linnaeanum, S. hispidum*, *S. torvum* SW, *S. scabrum* Mill	Fassuliotis and Dukes (1972); Pochard and Daunay (1977); McCammon and Honma (1983); Daunay et al. (1991); Collonnier et al. (2003a, b); Gousset et al. (2005); Zhuang and Wang (2009)

(continued)

Table 16.1 (continued)

Pathogens/pests	Resistant species	References
Colletotrichum coccodes	*S. linnaeanum* Hepper and Jaeger	Daunay et al. (1991)
Phytophthora parasitica	*S. aethiopicum* L. gr *aculeatum*, *S. torvum* SW.	Beyries et al. (1984)
Cercospora solani	*S. macrocarpon* L.	Madalageri et al. (1988)
Bacteria		
Ralstonia solanacearum	*S. capsicoides* All., *S. sisymbrifolium* Lam. *S. sessiliflorum* Dun.	Beyries (1979); Mochizuki and Yamakawa (1979)
	S. stramonifolium, Jacq., *S. virginianum* L., *S. Aethiopicum*, *S. aethiopicum* gr *aculeatum*, *S. grandiflorum* Ruiz, *S. hispidum* Pers. *S. torvum* SW, *S. nigrum* L., *S. americanum*, *S. scabrum*	Messiaen (1989); Hebert (1985); Sheela et al. (1984); Daunay et al. (1991); Collonnier et al. (2001b, 2003a, b); Gousset et al. (2005)
Nematodes		
Meloidogyne spp.	*S. ciarum* Dun., *S. sisymbrifolium* Lam., *S. elagnifolium* Cav., *S. violaceum*, *S. hispidum* Pers., *S. torvum* SW	Sonawane and Darekar (1984); Fassuliotis and Dukes (1972); Di Vito et al. (1992); Verma and Choudhury (1974); Daunay and Dalmasso (1985); Messiaen (1989); Shetty and Reddy (1986); Daunay and Dalmasso (1985); Messiaen (1989); Shetty and Reddy (1986)
Insects		
Leucinodes orbonalis	*S. mammosum* L., *S. viarum* Dun., *S. sisymbrifolium* Lam., *S. incanum, S. aethiopicum* gr *aculeatum*, *S. grandiflorum S. aethiopicum* gr *aculeatum*, *S. grandiflorum*	Baksh and Iqbal (1979); Lal et al. (1976); Chelliah and Srinivasan (1983); Khan et al. (1978)
Epilachana vigintioctopunctata	*S. mammosum* L., *S. viarum* Dun., *S. torvum* SW.	Beyries (1979); Sambandam et al. (1976)
Aphis gossypii	*S. mammosum* L.	Sambandam and Chelliah (1983)
Tetranychus cinnabarinus	*S. mammosum* L., *S. sisymbrifolium* Lam., *S. pseudocapsicum* L.	Shalk et al. (1975)
Tetranychus urticae	*S. macrocarpon* L.	Schaff et al. (1982)
Viruses		
Potato virus Y	*S. linnaeanum* Hepper and Jaeger	Horvath (1984)
Eggplant mosaic virus	*S. hispidum* Pers.	Rao (1980)
Others		
Mycoplasma (little leaf)	*S. hispidum* Pers., *S. aethiopicum* L. gr *aculeatum*, *S. viarum* Dun., *S. torvum* SW	Rao 1980; Khan et al. (1978); Charkrabarti and Choudhury (1974), Datar and Ashtaputre (1984)

bacterial and fungal wilts and nematode as well as, respectively, to the insect *L. orbonalis* and *E. vigintioctopunctata*.

In eggplant, wild relatives have been shown as an important source for tolerance to abiotic stresses and other agronomically important traits. High tolerance to frost was reported in *S. grandiflorum*, *S. mammosum* and *S. khasianum* (Baksh and Iqbal 1979), and tolerance to drought has been reported in *S. macrocarpon* L. while tolerance to salinity in *S. linnaeanum* (Daunay et al. 1991). *S. torvum* is one of the eggplant relatives widely studied because it exhibited high tolerance to salts and a low translocation of cadmium in the shoot. Thanks to these characters and also to the resistance to many soilborne diseases, *S. torvum* has been widely utilised as a rootstock (Bletsos et al. 2003). With regard to salt tolerance, eggplant grafted onto *S. torvum* showed more vigorous growth and less adverse effects of salinity. Various physiological and biochemical attributes (shoot length and stem width, chlorophyll pigments, proline content and activities of peroxidase (POD), polyphenoloxidase (PPO) and phenylalanine ammonia-lyase (PAL)) of grafted eggplants resulted higher than those of self-rooted plants (Bai et al. 2005; Bao-li et al. 2010). Cadmium (Cd) concentration in eggplant fruits is reported to be drastically reduced by grafting onto *Solanum torvum*; a more detailed characterisation of the mechanism of Cd translocation in *S. torvum* with respect to cultivated eggplant revealed that although they displayed a similar rate of adsorption in the roots, the wild species showed a significant lower translocation of Cd in the stem, leaf and fruit. Thus, *S. torvum* has been proposed for soil phytoremediation (Mori et al. 2009). These responses prompted the study to identify the genes of *S. torvum* involved in the tolerance to the heavy metal Cd and also to *Verticillium* by transcriptome analyses (Wang et al. 2010a; Yamaguchi et al. 2010; Xu et al. 2012), and the pathogenesis-related gene *StDAHP* was cloned following inoculation with *Verticillium* (Wang et al. 2010b).

In most of the work aimed to assess the response of the allied species to the biotic and abiotic stresses, often, a single or a few accessions were analysed. It is worth to point out that the allied and wild species probably display an even huge allelic variation for the useful traits searched because they have been mainly subjected to environmental selection including the indirect effect of the human activities and not to the bottleneck of domestication. Therefore the collections of wild and allied species need to be implemented and also to be better characterised for their biological and biochemical properties; with regard to eggplant only a few example can be reported, like *S. torvum* (Gousset et al. 2005) and *S. aethiopicum* (Sunseri et al. 2010).

16.4 Introgression of Alien Genes from Allied and Wild Species

16.4.1 Conventional Breeding Approach

The use of conventional breeding methods to introgress the alien traits of interest into cultivated lines of eggplant has been performed only sporadically through interspecific crosses because of the presence of sexual barriers between *S. melongena*

and its related wild species (Ano et al. 1991; Bletsos et al. 1998). The capability of eggplant to cross to species of other genera or subgenera is very low (Daunay et al. 1991). This may result from the lack of genetic information about the crossing partners or due to evolutionary divergence, which is known as incongruity (Franklin et al. 1995). Attempts at more distant crosses as that of *S. melongena* with *S. lycopersicon* resulted in sterile hybrids (Rao 1979).

Most of the published works about the sexual interspecific hybridisations between eggplant and its allied and wild relatives are listed in Table 16.2. More than 25 species have been employed in attempts to crossing with *S. melongena* in order to transfer useful traits. Only a limited number of them (*S. incanum* L., *S. linnaeanum*, *S. macrocarpon*, *S. aethiopicum* L., *S. viarum* and *S. virginianum*) were able to develop fertile or partially fertile F_1 hybrids which were successfully backcrossed to the cultivated *S. melongena*. Interspecific hybrids are also commercially employed as rootstock (e.g. cv "Taibyo VF" from a cross between eggplant and *S. grandiflorum*; Hasnumnahar et al. 2012) especially in Japan in field infested by fungal and bacterial wilt. It has been reported that interspecific hybrids with the wild relative *S. incanum*, which is more easily crossable with cultivated eggplant, display an acceptable level of tolerance to nematodes and produced fruits of good apparent and compositional quality compared to *S. torvum* (Gisbert et al. 2011).

The allied species, *S. linneanum* (=*S. sodomaeum*), which is efficiently cross compatible with eggplant has been used in development of mapping population for construction of a molecular map (Doganlar et al. 2002). Subsequently, it has been used in identification of QTLs for qualitative and quantitative traits (Frary et al. 2003). This species (Fig. 16.1a) has also been used to introgress the alien genes controlling to *Verticillium* wilt following backcross breeding, which resulted in the development of several wilt tolerant lines of eggplant (Acciarri et al. 2004). Also *S. tomentosum* has been successfully employed for alien genes introgression due to its partial fertility with cultivated species (Fig. 16.1b). The partially fertile hybrid obtained between this wild species and eggplant has been backcrossed with cultivated species for the improvement of agronomical and fruit quality traits (unpublished).

Most of the wild relative species, particularly *S. sisymbrifolium* and *S. torvum*, possess a large number of resistance traits to serious diseases of eggplant. However crossing of these species with cultivated eggplant gave no hybrids at all or only partially fertile hybrids through embryo rescue (Daunay and Lester 1988; Bletsos et al. 1998). Different eggplant lines showed a diverse capacity to be crossed with a given allied or wild relative and, sometimes, successful hybridisation of wild species with cultivated species had been obtained when wild species were used as female or *vice versa* (Rao 1979). For example, the interspecific hybrid with *S. anomalum* and *S. indicum* can produce seeded fruits only when they are employed as male parent and not as female (Behera and Singh 2002). Therefore, the employment of different accessions of the same wild relative with different lines of eggplant may improve the chance to obtain fertile interspecific hybrids. Interspecific crossing barrier may also be broken down by making several attempts of crossing to the same flowers along different days. A combination of the above approaches has allowed to obtain

Table 16.2 Interspecific crosses between the cultivated eggplant (*S. melongena* L.) and other *Solanum* species

Wild species	Status of crossability with cultivated species	References
S. melongena	Fertile F_1 plants	Pearce (1975)
S. incanum	Fertile F_1 plants	Pearce (1975); Behera and Singh (2002); Vilanova et al. (2010)
S. campylacanthum	Partially fertile F_1 plants	Pearce (1975)
S. linnaeanum (*S. sodomeum*)	Partially fertile or sterile F_1 plants	Pearce (1975); Pochard and Daunay (1977); Acciarri et al. 2004
S. macrocarpon	Partially fertile/sterile F_1 plants	Pearce (1975); Bletsos et al. (2004); Gowda et al. (1990)
S. marginatum	Partially fertile F_1 plants	Pearce (1975)
S. verginianum	Partially fertile F_1 plants or no F_1 plants	Pearce (1975); Rao (1979); Isshiki et al. (2000)
S. sodomeum	Fertile F_1 plants	Tudor and Tomescu (1995); Acciarri et al. (2004); Doganlar et al. (2002)
S. anomalum	Fertile F_1 plants	Behera and Singh (2002)
S. aethiopicum (gr. kumba, gr gilo *gr aculeatum*)	Partially fertile F_1 or sterile F_1 plants	Pearce (1975); Rao and Baksh (1979); Isshiki and Taura (2003); Prohens et al. (2012)
S. anguivi	Partially fertile F_1 plants	Pearce (1975)
S. cinereum	Sterile F_1 plants	Pearce (1975)
S. pyracanthos	No F_1 plants	Pearce (1975)
S. rubetorum	Partially fertile F_1 plants	Hassan (1989)
S. tomentosum	Partially fertile F_1 plants	Pearce (1975)
S. trilobatum	No F_1 plants, no seeds	Rao and Rao (1984)
S. violaceum	Partially fertile F_1 plants	Bulinska (1976); Isshiki and Kawajiri (2002)
S. hispidum	Partially fertile F_1 plants	Khan (1979); Magoon et al. (1962), Rao (1980)
S. torvum	Partially fertile or sterile or F_1 plants after embryo rescue	Pearce (1975), Bulinska (1976); McCammon and Honma (1983); Daunay et al. (1991); Bletsos et al. (1998)
S. capsicoides	No F_1 plants	Pearce (1975)
S. mammosum	No F_1 plants or abnormal seeds	Sambandam et al. (1976)
S. viarum = *khasianum*	Sterile F_1 plants after embryo rescue	Pearce (1975); Sharma et al. (1980)
S. grandiflorum	Partially fertile F_1 plants	Rao (1979)
S. lidii	Partially fertile F_1 plants	Hassan (1989)
S. sisymbrifolium	Sterile F_1 plants after embryo rescue	Sharma et al. 1984; Bletsos et al. (1998)
S. campanulatum	No F_1 plants or abnormal seeds	Pearce (1975)
S. stramonifolium	No F_1 plants, no seeds	Nishio et al. (1984)

interspecific hybridisation of *S. melongena* with *S. khasianum* (=*S. viarum*) and *S. sisymbrifolium* by using in vitro embryo rescue. In this case, it could only be possible when the wild relatives *S. khasianum* and *S. sisymbrifolium* were employed as female and male parent, respectively, during crossing with eggplant (Sharma et al. 1980, 1984).

The infertility of interspecific hybrids between *S. melongena* and others *Solanum* could be associated to self-incompatibility due to the wild parent, being the eggplant self-compatible (Daunay et al. 1991). However, the sterility is often attributable to lack of affinity of the genomes involved in the cross causing difficulty in correct pairing of chromosomes at the meiosis and the formation of irregular and sterile microspores and egg-cells. Reduced fertility or infertility is a common phenomenon in the interspecific hybrids of eggplant with other *Solanum* species (e.g. *S. macrocarpon*, *S. linneanum*) belonging to the *Melongena* section as well (Bletsos et al. 2004; Acciarri et al. 2007). However, fertility may be significantly improved or restored by doubling the ploidy level of the diploid (2x) hybrid, because synthetic amphidiploid has a more balanced chromosomes pairing during meiosis. For example, fertility restoration has been reported in the sexual hybrid between eggplant and *S. aethiopicum* (Isshiki and Taura 2003). Similarly, male-sterile plants were selected following the sexual hybridisation of eggplant with *S. violaceum* (Isshiki and Kawajiri 2002). Isshiki and collaborators employed *S. virginianum*, *S. aethiopicum* Aculeatum group and *S. grandiflorum* cytoplasms to develop male-sterile lines of eggplant through sexual hybridisation; the relative species were used as female parent and *S. melongena* as male to obtain the F_1 interspecific hybrids and the successive backcrossed progenies were selected for male sterility. Functional (indeishent anthers) and cytoplasmic (non-forming pollen) male-sterile introgression lines were developed. Furthermore, in the case of *S. grandiflorum*-derived cytoplasmic male sterility, two independent dominant restorer fertility (*Rf*) genes have been discovered to control pollen formation (Khan and Isshiki 2008, 2010; Hasnumnahar et al. 2012).

16.4.2 Somatic Hybridisation

Plant regeneration from protoplast has been efficiently set up in eggplant (Sihachakr and Ducreux 1987). This has provided an opportunity to apply the somatic hybridisation technology for introgression of alien genes in order to make genetic improvements of eggplant (Sihachakr et al. 1994; Collonnier et al. 2001a; Kashyap et al. 2003; Rajam and Kumar 2006). It offers the possibility of overcoming sexual barriers or improving the fertility of interspecific hybrids with respect to the correspondent ones which are obtainable by conventional breeding methods (Sihachakr et al. 1994). Moreover, with respect to sexual hybridisation, somatic fusion may lead to new and unique nucleo-cytoplasmic combinations with higher genetic variability from the mixture of nuclear and cytoplasmic genomes of the fusion partners. It is also associated to increase the possibility of obtaining somaclonal variants. For example, a salt-resistant line of eggplant has been isolated from cell culture in a medium containing 1 % sodium chloride (Jain et al. 1988). In field trials, potential useful genetic variation for agronomic traits was observed in both embryogenic and androgenic eggplant lines (Rotino 1996).

In eggplant, the first successful somatic hybridisation was obtained with *S. sisymbrifolium*. These somatic hybrids were resistant to root-knot nematodes and potentially resistant to spider mites. However, the strong sterility of these hybrids has

prevented their practical utilisation (Gleddie et al. 1986). Other tetraploid somatic hybrids obtained with *S. sisymbrifolium* have shown resistant to *Ralstonia solanacearum* and culture filtrate of *V. dahliae* in in vitro tests (Collonnier et al. 2003a). The somatic hybrids with *S. khasianum*, a relative displaying resistance to shoot and fruit borer, despite it showed a promising percentage (12 %) of viable pollen, produced only seedless parthenocarpic fruits (Sihachakr et al. 1988). Resistance to the herbicide atrazine was achieved in somatic hybrids of eggplant with *S. nigrum* (Guri and Sink 1988b; Sihachakr et al. 1989). *S. torvum* was also widely employed in different fusion experiments; the somatic hybrids were found resistant to nematodes, partially resistant to spider mite and displayed good levels of resistance to *R. solanacearum* and culture filtrate of *V. dahliae*. However, greenhouse-grown plants resulted sterile (Guri and Sink 1988a; Sihachakr et al. 1989; Collonnier et al. 2003b). *S. torvum* was also employed to obtain highly asymmetric somatic hybrids tolerant to *Verticillium* wilt, which displayed a morphology similar to the cultivated eggplant (Jarl et al. 1999). The asymmetric hybrids were obtained following the X-ray treatment of the donor genotypes (*S. torvum*) in order to obtain hybrids bearing few chromosome fragments from the donor parent associated with a complete set of chromosomes from the other parent (Jones 1988; Sihachakr et al. 1994). Somatic hybridisation was accomplished between eggplant and a sexual hybrid of tomato with its wild relative, *Lycopersicon pennellii*, producing only hybrid calli with few leaf primordial (Guri et al. 1991). Subsequently highly asymmetric hybrid plants were regenerated after irradiation of one fusion partner with γ-rays (Liu et al. 1995; Samoylov and Sink 1996; Samoylov et al. 1996). Fertile tetraploid somatic hybrids were also obtained between eggplant and *S. marginatum* in view of converting the cultivated eggplant to an arborescent perennial species; the selfed progenies maintain the arboreous character but showed a low frequency of tetravalent formation during microsporogenesis (Borgato et al. 2007).

Somatic hybridisation with *S. aethiopicum* is an excellent example of successful incorporation of somatic hybrids in breeding programmes (Fig. 16.2a). The tetraploid somatic hybrid was obtained through electrofusion to introgress the traits of resistance to bacterial and fungal wilts from *S. aethiopicum* into cultivated eggplant (Daunay et al. 1993; Rotino et al. 2001). Most of the hybrids had the eggplant ctDNA. Field evaluation showed that the tetraploid somatic hybrids had a much better pollen viability and, most important, gave a high yield of fruits (up to 9 kg/plant) while the diploid sexual hybrids produced only few seedless fruits. The somatic hybrids resulted highly resistant to bacterial wilt in inoculation tests carried out in a contaminated field (Rajam et al. 2008). Similarly, other fertile somatic hybrids of eggplant with *S. aethiopicum* gr. *aculeatum* were resistant to *Fusarium* wilt (Fig. 16.2b; Rotino et al. 1998, 2001). These results support the previous reports that the tetraploid status significantly improves the fertility of the interspecific hybrid which produces seeded fruits. However, in order to incorporate the somatic hybrid in a practical eggplant breeding programme for introgression of traits of interest, two essential prerequisites still need to be accomplished beyond fertility: (1) occurrence of genetic recombination between the genomes of the two species and (2) reduction of the ploidy level to that of recurrent parent because generally the

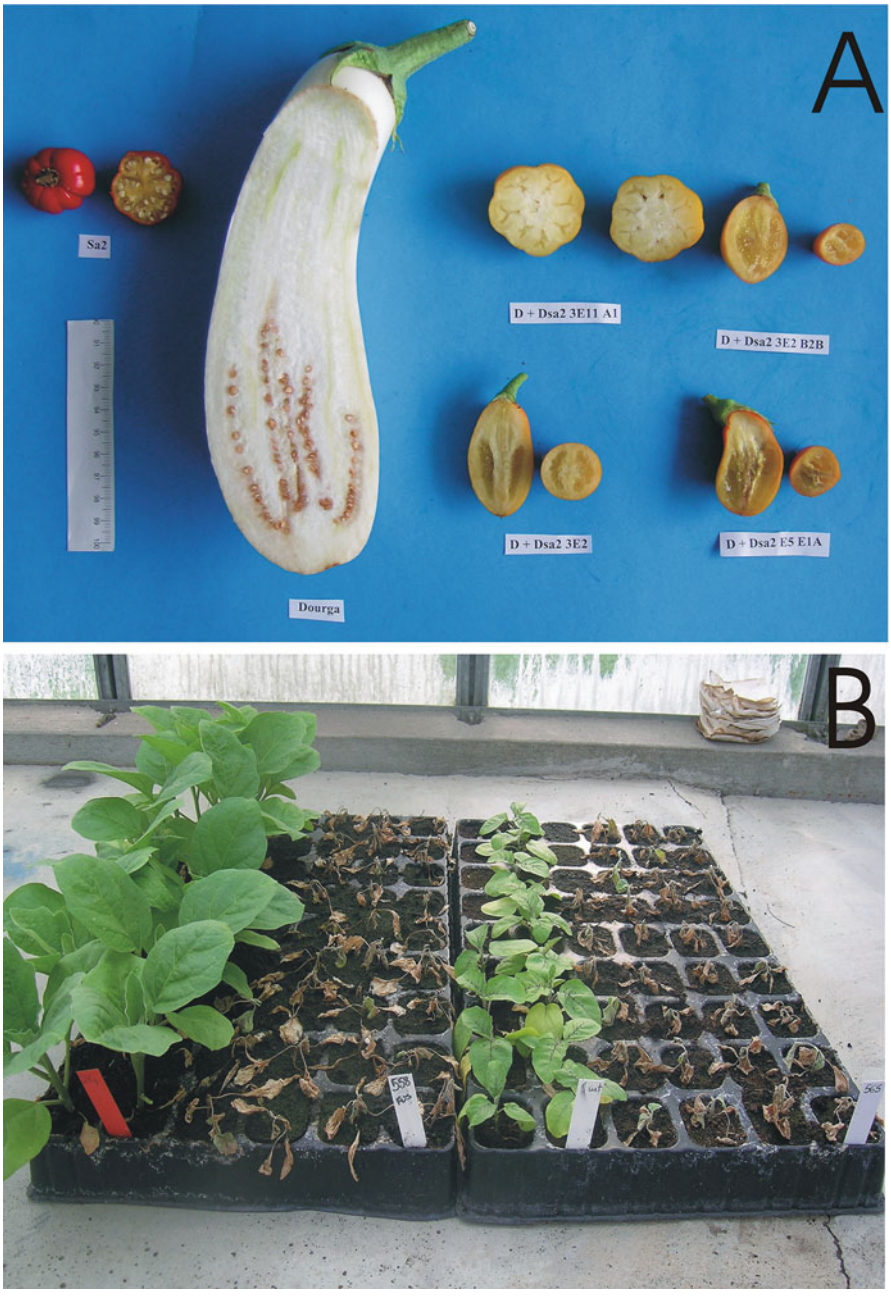

Fig. 16.2 Fruits from interspecific hybridisation of eggplant with allied species. (**a**) Somatic hybridisation between *S. melongena* and *S. integrifolium*; from *left*: *S. aethiopicum*, eggplant cv Dourga and four somatic hybrids. (**b**) Phenotypical evaluation of the progenies for the resistance to *Fusarium oxysporum* scored after 30 days from inoculation with the fungus; from the *left*, an introgression line from a somatic hybrid with *S. integrifolium* (resistant), a recurrent genotype of eggplant (completely dead), *S. integrifolium* (donor, resistant), another recurrent genotype of eggplant (completely dead)

somatic hybrids possess the sum of the genomes of the involved species. In case of first point, synthetic amphidiploids from sexual hybridisation are capable to form multivalents at meiosis leading to segregation in their selfed progenies (Isshiki et al. 2000; Isshiki and Taura 2003). However, the presence of multivalents at meiosis is necessary but not sufficient condition for genetic recombination because crossing over does not always occur after chromosome pairing. In relation to the second point, eggplant is quite responsive to the tissue and organ culture including the regeneration of androgenetic plants from microspore mainly through anther culture (Rotino 1996). This technology has been efficiently exploited to regenerate dihaploid plants either from the somatic hybrids (Rizza et al. 2002) or from a tetraploid BC_1 backcross (Rotino et al. 2005). Occurrence of an effective genetic recombination was demonstrated in the population of androgenetic dihaploids derived from anther culture of the somatic hybrid between *S. melongena* and *S. aethiopicum* gr. *gilo* (Rizza et al. 2002). A population of 71 dihaploids was elegantly employed to clearly demonstrate that crossovers (i.e. random chromatid segregation) occurred between the chromosomes of the two species by following the segregation of 280 ISSR markers and 5 isoenzymatic loci (Toppino et al. 2008a). The dihaploids, which were highly heterozygous, displayed a wide range of morphological variation and segregated for resistance to *Fusarium*. These variations were never showed off by the selfed progenies of the tetraploid somatic hybrids, which appeared phenotypically uniform and were all resistant to *Fusarium* (Rotino et al. 2005). Although first backcrossing of the dihaploids with the recurrent eggplants has been obtained with a certain difficulty, in subsequent backcross generations, morphology and fertility improved progressively (Rotino et al. 2005). Thus, these BC_1-derived dihaploids have been shown more useful in the breeding programme for faster and straightforward selection of introgression lines with resistance to *Fusarium* (Fig. 16.2b). The development of advanced backcross introgression resistant lines has been used to identify the molecular marker linked to resistance that behaves as a dominant monogenic trait (Toppino et al. 2008b).

Another interspecific hybrid using *S. viarum* has been successfully backcrossed to eggplant and after selfing for nine generations resulted in the identification of two lines having higher yield, good fruit quality and high tolerance to the devastating insect fruit and shoot borer (Pugalendhi et al. 2010). Further biochemical analyses of these lines showed that the tolerant introgression lines had increased activity of polyphenoloxidase and peroxidase and higher level of solasodine and total phenol (Prabhu et al. 2009). Backcrossed progenies obtained from the sexual hybrid of eggplant with *S. aethiopicum* gr. *aculeatum* showed the resistance to *Fusarium* (Zhuang and Wang 2009). Similarly backcross progenies (BC_1) derived from the crossing between the *S. incanum* gr. C and eggplant have been used to construct an interspecific linkage map and development of introgression lines (Vilanova et al. 2010). Interspecific hybridisation has also been accomplished in the less cultivated *S. aethiopicum* Kumba group, and its BC_1 progenies have been analysed in view of their possible exploitation for the improvement of eggplant (Prohens et al. 2012).

16.4.3 Linkage Drag of Undesirable Traits During Alien Gene Introgression

Introgression of alien genes from allied and wild species may result in linkage drag of undesirable agronomical traits such as susceptibility to (new) pest and diseases and/or a high level of unsafe compounds such as glycoalkaloids along with desirable traits. For example, the use of wild species *S. torvum* carries a very high susceptibility to *Colletotrichum gloeosporioides* (Collonnier et al. 2001a). The detailed analyses of glycoalkaloid levels in *S. macrocarpon* and *S. aethiopicum*, which have been shown potential genetic resources for improvement of eggplant, revealed that *S. macrocarpon* fruits had values 5–10 times higher than those considered to be safe in foods and might not be considered suitable for human consumption while fruits of *S. aethiopicum* presented values similar to those of *S. melongena* and could be considered as safe for consumption (Sanchez-Mata et al. 2010). *S. sodomaeum* also produces fruits very rich in glycoalkaloids; this species is very close to eggplant as they both belong to the section *Melongena* of Solanaceae and interspecific hybrids between them have been obtained and incorporated in breeding programmes (see above). A biochemical characterisation of the health-related compounds including glycoalkaloids (solamargine and solasonine) was carried out in advanced introgression lines from *S. sodomaeum* and also of *S. aethiopicum* gr *gilo* and gr. *aculeatum* (=*S. integrifolium*), in the eggplant recurrent genotypes and the three allied species (Mennella et al. 2010). This study demonstrated that most of the introgression lines, obtained after several cycles of backcross with eggplant, showed biochemical composition and functional properties similar to that of the recurrent eggplants, even though the selection was made only on the basis of morphological traits of interest and tolerance trait to *Verticillium*. However, it is to point out that some introgression lines from *S. sodomaeum* still presented significantly higher values (three to six times) than the recurrent eggplant (Mennella et al. 2010). Therefore, it could be advisable, as first step in an introgression breeding programme, to check the biochemical composition of the fruits in the allied and wild parents of the interspecific hybrids; then, according to the results obtained and the goals of the programme, the biochemical analyses might be also employed as a selection tool or limited to the last characterisation of the final genetic materials obtained.

16.5 Genetic Engineering

Eggplant, as many other members of the Solanaceae family, is highly responsive to genetic transformation (Rajam et al. 2008). The main achievements of genetic transformation via *Agrobacterium* are listed in Table 16.3. Transgenic plants were firstly

Table 16.3 Genetic transformation in eggplant (*Solanum melongena* L.)

Explant	Gene	Accomplishment	References
Leaf	*npt*II	Transgenic plant using a cointegrate vector	Guri and Sink (1988a)
Cotyledon/leaf	*npt*II	Stable transformation with a binary vector	Filippone and Lurquin (1989)
Leaf	*npt*II, *cat*	Transformation efficiency of 7.6 %	Rotino and Gleddie (1990)
Leaf	*npt*II, *gus*	In vivo selection of *npt* II gene in transgenics	Sunseri et al. (1993)
Cotyledon	*npt*II, *gus*	Transformation through organogenesis and embryogenesis	Fári et al. (1995); Akhter et al. (2012)
Hypocotyl	*Bt (cryIIIB)*	Resistance to CPB was not observed	Chen et al. (1995)
Cotyledon/Leaf	Mutagenised *Bt (cryIIIB)*	Resistance to CPB; Field trials; strategy of Bt-resistant eggplant deployment; spider mites preference; wide ecological study	Arpaia et al. (1997); Iannacone et al. 1997; Acciarri et al. (2000); Mennella et al. (2005); Rovenska´ et al. (2005); Arpaia et al. (2007)
Leaf	*Bt (cryIIIB)*	Effect of hormones and antibiotics on efficiency of transformation	Billings et al. (1997)
Leaf	Synthetic *Bt (cry IIIA)*	Resistance to CPB	Hamilton et al. (1997); Jelenkovic et al. (1998)
Cotyledon	Synthetic *Bt (cry1Ab)*	Resistance to *Leucinodes orbonalis*	Kumar et al. (1998)
Leaf	*Luc*	Stability of luciferase gene expression	Hanyu et al. (1999)
Cotyledon	*PAtgtrp-5::GUS*	Studies about factors influencing transformation efficiency	Magioli et al. (2000)
Cotyledon/leaf	*DefH9-iaaM*	Parthenocarpic transgenic plants; green house trial; green-house and open-field trials; biochemical and technological evaluation of parthenocarpic fruits	Rotino et al. (1997a, b); Donzella et al. (2000); Acciarri et al. (2002); Maestrelli et al. (2003)
–	Yeast Δ-9 desaturase	Increased resistance to *Verticillium* wilt	Xing and Chin (2000)
Cotyledon	*Mtl*D	Tolerance against osmotic stress	Prabhavathi et al. (2002); Prabhavathi and Rajam (2007a)
Root	*npt*II	Efficient and stable transformation	Franklin and Sita (2003)
Leaf, cotyledon, hypocotyl	*Gfp:gus,hpt, VirE:LacZ*	Increased transformation efficiency	Kumar and Rajam (2005)
Cotyledon	*Mi-1.2*	Resistance to root-knot nematode	Goggin et al. (2006)
Cotyledon	*Oryzacystatin*	Resistance to aphids	Ribeiro et al. (2006)

Leaf	*Dm-AMP1*	Resistance to *Botrytis cinerea*; release of the defensin in their root exudates; inhibitory effect on *Verticillium albo-atrum*	Turrini et al. (2004)
Cotyledon, hypocotyl	*npt*II	Influence of growth regulators and explants type on Agrobacterium transformation; hypocotyl regenerate better than cotyledons	Prakash et al. (2007a, b)
	Bt (Cry2Ab)- nptII-gus	Antibiotic marker-free transgenic plant obtained by using *A. tumefaciens* bearing two T-DNA plasmids	Narendran et al. (2007)
Hypocotyl	*Cry1Ac*	Complete resistance towards EFSB	Pal et al. (2009)
Hypocotyl	*Barnase, Cre/loxP*	A combined system to induce a restorable male sterility in eggplant	Cao et al. (2010)
Stem	*aadA*	Optimisation of plastid transformation protocol in eggplant	Singh et al. (2010)
Leaf, cotyledon	*SmTAF10,SmTAF13*	Developing of a reversible male sterility system	Toppino et al. (2010)
Cotyledon	*CMV-CP*	Resistance to CMV	Pratap et al. (2011)
Leaf, cotyledon	*Cry1Fa1*	Resistance to BFSB	Shrivastava et al. (2011)
Shoot tip	*DREB1A*	Moisture stress tolerant eggplant	Sagare and Mohanty (2012)

*npt*II, *neomycin phosphotransferase* II; *hpt*, *hygromycin phosphotransferase*; *cat*, *chloramphenicol acetyltransferase*; *Bt (cry IIIB)*, *Bacillus thuringiensis cry IIIB*; *Bt (cry IIIA)*, *Bacillus thuringiensis cry IIIA*; *Bt (Cry 1Ab)*, *Bacillus thuringiensis cry 1Ab*; *gus*, *β-glucuronidase*; *luc*, *luciferase*; *pAtgrp-5,* regulatory region of the *Arabidopsis thaliana* glycine rich protein 5; *DefH9-iaaM*, regulatory region of the *DEFICIENS 9* gene from snapdragon and the auxin-synthesising gene coding region *(IaaM)* from *Pseudomonas syringae* pv. *savastanoi; mtl*D, bacterial *mannitol-1-phosphodehydrogenase* gene; *Dm-AMP1*, *antimicrobial defensin from Dahlia merckii gene; Bt (Cry 2Ab)*, *Bacillus thuringiensis cry 2Ab; Barnase,* cell lethal gene*; Cre/loxP* site-specific recombination system; *aadA* gene for resistance to spectinomycin and streptomycin. *SmTAFs*, *Solanum melongena TBP-associated factors* gene. *CMV-CP*, *Cucumber mosaic virus coat protein* gene. *Cry1Fa1*-synthetic Bt gene. *DREB1A*, *Arabidopsis dehydration-responsive element* gene

obtained by Guri and Sink (1988c), using leaf explants and a cointegrate vector carrying the *npt*II gene; in the following years, Filippone and Lurquin (1989) reported stable callus transformation and Rotino and Gleddie (1990) obtained transgenic plants using a binary vector. Since then, different *Agrobacterium* strains harbouring binary vectors were employed to raise transgenic eggplants carrying kanamycin resistance (*npt*II) as selective agent and different reporter genes, including *gus* (*β-glucuronidase*), *cat* (*chloramphenicol acetyl transferase*) and *luc* (*luciferase*).

Resistance to Colorado potato beetle (*Leptinotarsa decemlineata* Say) (CPB), which is a serious pest for eggplant cultivation in Europe and America as it develops resistance to synthetic insecticides (Arpaia et al. 1997), has been pursued by a number of groups. Chen et al. (1995) produced transgenic eggplant lines with the introduction of *Bacillus thuringiensis* (Bt) genes, but resistance to CPB was not observed. Later, different groups were able to obtain lines resistant to CPB by using mutagenised versions of *cryIIIB* (Arpaia et al. 1997; Iannacone et al. 1997) or a synthetic version of *cryIIIA* Bt genes by adjustment of the codon usage for expression in dicots (Hamilton et al. 1997; Jelenkovic et al. 1998). Field trials demonstrated high levels of resistance in transgenic plants for the mutagenised Bt *cryIIIB* gene, which ensured a significantly higher production than untransformed controls without detrimental effects on some nontarget arthropods (Acciarri et al. 2000). Moreover, strategies for field deployment of eggplant-Bt expressing were also evaluated (Mennella et al. 2005). A study in a greenhouse revealed that the transformed *cryIIIB*-eggplants were a food more preferred for spider mites and simultaneously less preferred by predatory mites. Such a shift in the preference of both the spider mites and their predators could result in lower effectiveness or even failure of biological control (Rovenska´ et al. 2005). A further wide open field 3-year study on species assemblage of herbivorous and insect biodiversity in experimental fields of Bt *cryIIIB*-expressing lines evidenced a comparable nontarget species assemblage between transgenic and near-isogenic eggplant cultivation areas (Arpaia et al. 2007).

Resistance to *Leucinodes orbonalis*, the eggplant fruit and shoot borer (EFSB), which is a lepidopteran insect very destructive in Asian countries, was firstly obtained using a synthetic *cry1Ab* gene modified for rice codon usage (Kumar et al. 1998). Antibiotic marker-free transgenic plants resistant to EFSB were also obtained by using *A. tumefaciens* bearing two T-DNA plasmids containing the Bt *cry2Ab* gene and the *nptII* plus the *gus* genes, respectively; *cry2Ab* and marker genes segregated independently in 44 % of the primary transformants demonstrating that the T-DNA insertions were unlinked (Narendran et al. 2007). A *cry1Ac* Bt gene expressed in an elite eggplant line gave also a complete resistance towards EFSB (Pal et al. 2009). Recently, a synthetic Bt gene (*cry1F*) effective against *Leucinodes orbonalis* was introduced into eggplant by means of targeted homologous recombination in a gene of the anthocyanin pathway *F3G* (*flavonoid-3-glucosyltransferase*) without the aid of any recombinase gene, and out of 956 kanamycin-resistant plantlets, 2 had the expected insertion of the 35S-*cry1F* in the *F3G* gene (Shrivastava et al. 2011).

In several field experiments, Bt-eggplant effectively controlled EFSB; efforts to commercialise in India and the Philippines eggplant hybrids expressing Bt protein

have been done including extensive biosafety investigations, nutritional and substantial equivalence studies and relative toxicity and allergenicity assessment using animal models like Sprague Dawley rat, brown Norway rat, rabbit, fish, chicken and goat; however the final approval still has not been given (Kumar et al. 2011).

The transgenic eggplant expressing oryzacystatin, a cysteine proteinase inhibitor of rice, reduced the net reproductive rate, the instantaneous rate of population increase and the finite rate of population increase of the two aphids *Myzus persicae* and *Macrosiphum euphorbiae* with respect to the control line (Ribeiro et al. 2006). These results indicate that expression of oryzacystatin in eggplant has a negative impact on population growth and mortality rates of *M. persicae* and *M. euphorbiae* and could be a source of plant resistance for pest management of these aphids.

The tomato *Mi-1.2* gene which confers race-specific resistance against root-knot nematodes *(Meloidogyne* spp.), potato aphids (*Macrosiphum euphorbiae*) and the insect whiteflies (*Bemisia tabaci*) was successful transferred via *A. tumefaciens* transformation into eggplant and a susceptible tomato cultivar (Goggin et al. 2006). The transgenic eggplants expressing *Mi-1.2* displayed resistance to *Meloidogyne incognita* but not to *Macrosiphum euphorbiae*; on the contrary, the transgenic tomato was resistant to both nematode and aphids (Goggin et al. 2006). Thus, the tomato *Mi-1.2* resistance gene retains partial function when introduced into another related solanaceous species. The lack of resistance to insects in eggplant could be due, for example, to deficiencies in the *Mi-1.2* sequence translation or protein processing in eggplant leaves and/or to absence of conservation, between tomato and eggplant species, of factor(s) essential for aphid resistance (Goggin et al. 2006).

This result deserves a further consideration about the transferability of useful traits from one species to another through genetic engineering or by means of hybridisation. In the first case (based on the introduction of a single or a few genes), the knowledge of the genetic basis underlying the trait and, as evidenced for *Mi-1.2* gene, of its mechanism of action is an essential prerequisite. In the second case (based on interaction between entire genomes), theoretically, it is not necessary to have any genetic information about the trait of interest, provided that an efficient method to select the introgressed progenies for this trait is available.

Resistance to the *Cucumber mosaic virus* (CMV) was engineered by insertion of the coat protein (CP) of *CMV* gene under the control of the constitutive promoter *CaMV 35S*, independent primary T0 transgenics resulted either tolerant or completely resistant when challenged with CMV virus (Pratap et al. 2011).

Eggplant was also engineered for *Verticillium* wilt resistance by overexpression of a yeast Δ-9 desaturase gene, which increased the levels of 16:1, 18:1 and 16:3 fatty acids (Xing and Chin 2000). The transgenic eggplants showed improved resistance to *Verticillium* wilt and, when inoculated with the pathogen, displayed a marked increase in the content of 16:1 and 16:3 fatty acids; it was also shown that *cis*-Δ9 16:1 fatty acid inhibited *Verticillium* growth.

Resistance to the fungus *Botrytis cinerea* was achieved by constitutive expression of the antimicrobial defence gene *Dm-AMP1* from *Dahlia merckii*; the transgenic plants released the defensin in their root exudates that had an inhibitory effect

on the pathogenic *Verticillium albo-atrum* while did not interfere with the symbiotic arbuscular mycorrhizal fungus *Glomus mosseae* (Turrini et al. 2004).

The bacterial *mannitol-1-phosphodehydrogenase* (*mtlD*) gene, which is involved in the mannitol synthesis when expressed in eggplant, caused tolerance to osmotic stress induced by salt, drought and chilling (Prabhavathi et al. 2002). Interestingly, these transgenic plants expressing *mtlD* gene with mannitol accumulation also exhibited increased resistance against three fungal wilts caused by *Fusarium oxysporum, Verticillium dahliae* and *Rhizoctonia solani* under both in vitro and in vivo growth conditions. Mannitol levels could not be detected in the wild-type plants, but the presence of mannitol in the transgenics could be positively correlated with the disease resistance (Prabhavathi and Rajam 2007a, b). Further, various transgenic eggplants overexpressing the oat *arginine decarboxylase* gene-encoding enzymes in the polyamine metabolic pathway were also generated. These transgenic plants showed increased tolerance to multiple abiotic (salinity, drought, extreme temperature and heavy metals) as well as biotic (fungal pathogens) stresses (Prabhavathi and Rajam 2007a). Tolerance to moisture stress was accomplished by expression of the transcriptional activator controlling the expression of genes containing C-repeat/dehydration-responsive element (*DREB1A*) under the control of the stress inducible promoter *rd29A*; morpho-physiological and biochemical analyses were performed, and transgenic plants, when subjected to extended stress condition, were in normal condition while control plants were completely dried (Sagare and Mohanty 2012).

Transgenic eggplants with parthenocarpic fruits were also developed by manipulating the auxin levels during fruit development through the introduction of *iaaM* gene from *Pseudomonas syringae* pv. *savastanoi* under the control of the ovule-specific promoter *DefH9* from *Antirrhinum majus* (Rotino et al. 1997b). These transgenics produced seedless parthenocarpic fruits in the absence of pollination without the external application of plant hormones, even at low temperature, which normally prohibit fruit production in untransformed lines. Further, these transgenics exhibited significantly higher winter yields than the untransformed plants and a commercial hybrid under an unheated glasshouse trial (Donzella et al. 2000). Trials in open field for summer production and greenhouse for early spring production confirmed that transgenic parthenocarpic eggplant F_1 hybrids gave a higher production coupled with an improved fruit quality with respect to the untransformed controls (Acciarri et al. 2002). Quality parameters of frozen parthenocarpic fruits did not show any significant changes when compared to their controls (Maestrelli et al. 2003).

Recently, a reversible male sterility system was developed in eggplant (Toppino et al. 2010). An anther-specific artificial microRNA-mediated silencing of two endogenous *TBP-associated factors* (*TAFs*) encoding genes was employed to obtain male-sterile eggplants (Fig. 16.3). Recovery of male fertility was successfully triggered by the addition in the unique transformation construct of an ethanol-inducible alternative form of the *TAF* genes insensitive to the amiRNA-mediated silencing; short treatments with ethanol allowed the formation of viable pollen able to perform pollination (Toppino et al. 2010) (Fig. 16.3a–c). The stability and the efficiency of this recoverable system make it interesting in view of the development of a transgene containment system but can also be adopted as a useful tool for commercial hybrid seed production. By combining this system with induced parthenocarpy

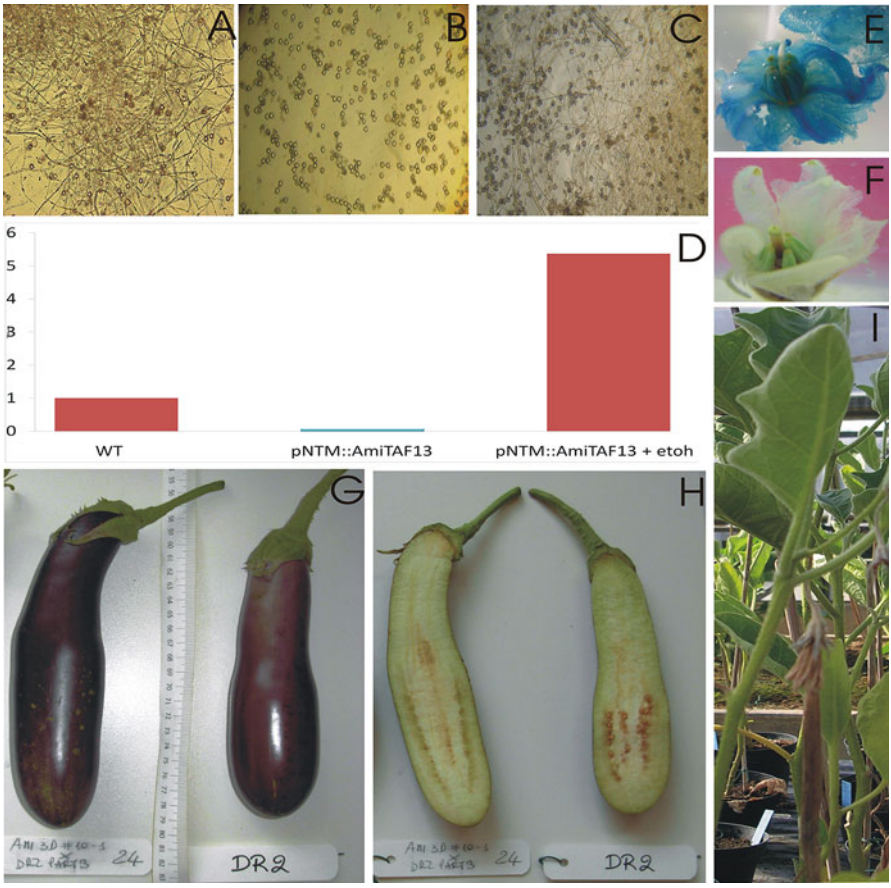

Fig. 16.3 Phenotypic and molecular evaluation of the eggplant lines transformed with the amiRNA targeting a TAF gene of eggplant. (**a**–**c**) Representative images of a pollen germination assay for wild-type and amiRNA-containing eggplant lines. (**a**) Wild-type germinating pollen. (**b**) Pollen of an eggplant transformed with the amiRNA construct, showing that pollen grains do not germinate. (**c**) Pollen of the same plant as shown in panel **b**, but after treatment of developing flowers with ethanol, pollen development and germination is restored. (**d**) Real-time analysis showing the TAF wild-type expression, the pNTM19: amiTAF13-mediated silencing of the TAF gene and the induction of the amiRNA-insensitive form of the TAF gene upon EtOH treatment of the same line. (**e**–**f**) Phenotypic evidence of the tissue-specific expression of the reporter gene GUS under the promoter pNTM (**f**), with respect to the systemic expression of the same reporter gene under constitutive 35S promoter (**e**). (**g–h**) Fruits from the cross between the amiRNA-transformed lines and parthenocarpic eggplant display identical phenotype with respect to the wild-type eggplant line DR2 but are completely seedless. (**i**) Inhibition of pollen fertility in the transformed lines of eggplant reflects in increment of the ratio of dried flowers on the plant

(Rotino et al. 1997b) (Fig. 16.3g, h), a novel example of complete transgene containment (male and female) in eggplant could be provided enabling biological mitigation measures for coexistence or biosafety purposes of GM crop cultivation. In addition, the growers could benefit of the increased yield of the parthenocarpic

male-sterile eggplants and of the improved quality of seedless fruit produced (Donzella et al. 2000; Acciarri et al. 2002; Maestrelli et al. 2003).

A combined system to induce a restorable male sterility in eggplant was also developed by utilising a cell lethal (ribonuclease) gene *Barnase* under the control of the *TA29* promoter, which ensures the expression specifically in the tapetum, coupled to the *Cre*/*loxP* system (Cao et al. 2010). The eggplant line "E-38" was transformed with *Cre* gene and the line "E-8" with the *TA29-Barnase* chimeric gene situated between *loxP* recognition sites for *Cre*-recombinase. Four T_0-plants with the *Barnase* gene proved to be male-sterile and incapable of producing viable pollen. The crossing of male-sterile Barnase plants with *Cre* expression transgenic eggplants resulted in site-specific excision with the male-sterile plants producing normal fruits since pollen fertility was fully restored in the hybrids when the Barnase gene was excised (Cao et al. 2010).

16.6 Conclusion

Alien gene introgression from wild species has been attempted using conventional breeding approaches. However, cross-incompatibility of most of the wild species has restricted their use in crop improvement, although different strategies have deployed to overcome these problems. However, relevant knowledge acquired on eggplant regeneration from different organs, tissues, cells and protoplasts has resulted development of somatic hybrids (Rajam et al. 2008). Further development of new tools and techniques will certainly improve the use of wild species in alien gene introgression.

Considerable progress has been gathered in the genetic improvement of eggplant by exploitation of the biotechnological tools of androgenesis and, to certain extent, of genetic transformation and somatic hybridisation. However, the general controversial acceptance of the GM plants has stopped so far the possible practical utilisation of trans- and cis-genesis in eggplant, even though this species could greatly take advantage of these technologies for transferring foreign genes. Thus, it has become more urgent to fill in the gap about the understanding of the *S. melongena* genome structure and organisation through the development of molecular tools which may allow to drive the breeding, including the transfer of alien genes from its allied and wild relatives.

The first well-saturated intraspecific maps have been recently developed (Fukuoka et al. 2012; Barchi et al. 2012a), making available to the scientific community DNA sequence databases and collections of expressed sequence tags (ESTs) for genomic analysis and markers mining. These tools have facilitated the localisation of the first quantitative trait loci (QTLs) of agronomic interest (Miyatake et al. 2012; Barchi et al. 2012b; Lebeau et al. 2012). The huge variation shown by the progenitors, allied and wild relatives of *S. melongena* is still untapped and could be better evaluated and employed by the use of molecular tools and introgression lines. Introgression lines (ILs) are more powerful in QTL identification of favourable alleles of alien

QTL because they carry a single introgressed region and thus the phenotypic variation in these lines can be associated with individual introgression segments. For precise introgression of these favourable alleles into cultivated eggplant, marker-assisted selection will play an increasing crucial role as the map positions and markers linked to the QTLs will allow to plan optimal breeding strategies.

The recent released sequence of the potato (The Potato Genome Sequencing Consortium 2011) and tomato (The Tomato Genome Consortium 2012) genomes certainly will facilitate the genetic recognition of the putative synthetic genetic regions, the development of highly saturated comparative maps, and the identification of useful orthologous regulatory and structural genes in wild species eggplant. Until the entire eggplant genome will be deciphered, the availability of such heterologous genomic knowledge will make anyhow easier the development of molecular markers and isolation useful genes in the background of wild relatives. These genes can also be exploited through genetic transformation. Allelic variation underlying the traits of interest for biotic and abiotic stress resistance and other useful agronomical traits in *Solanum* relatives will be utilised using recent molecular tools and techniques.

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