Satbir Singh Gosal · Shabir Hussain Wani
Editors

Biotechnologies of Crop Improvement, Volume 3

Genomic Approaches

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Editors
Satbir Singh Gosal
School of Agricultural Biotechnology
Punjab Agricultural University
Ludhiana, Punjab, India

Shabir Hussain Wani
Mountain Research Centre
for Field Crops, Khudwani
Sher-e-Kashmir University of Agricultural
Sciences and Technology of Kashmir
Srinagar, Jammu and Kashmir, India

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This book is dedicated to Dr. Gurdev Singh Khush – an acknowledged Hero of Rice Revolution.

Dr. Gurdev Singh Khush was born in a small village Rurkee of district Jalandhar, Punjab, India. He obtained B.Sc. degree in 1955 from the Government Agricultural College (now Punjab Agricultural University), Ludhiana, and Ph.D. in 1960 from the University of California, Davis, USA. Dr. Khush is a world-renowned geneticist and plant breeder who worked at the International Rice Research Institute (IRRI), Manila, Philippines, for 35 years. He has made enormous contributions to rice genetics and breeding. He is considered the main architect of green revolution in rice. More than 300 rice varieties (including mega-varieties IR36 and IR64), developed under his leadership, have been grown on 60% of the world's rice land. World rice production increased from 257 million tons in 1966 to 600 million tons in 2000, and most of the rice-growing countries became self-sufficient in rice. He has visited more than 60 rice-growing countries to observe rice research and production systems and served as a consultant to many national rice improvement programs. For his contribution to rice research and production, Dr. Khush has been honored with the World Food Prize, which is regarded as equivalent to the Nobel Prize in Agriculture. Other international awards received by him include the Japan Prize, Rank Prize from the UK, Wolf Prize in Agriculture from Israel, Golden Sickle Award from Thailand, and Mahathir Science Award from Malaysia. He has been bestowed honorary doctorate degrees by 16 universities, including the Punjab Agricultural University (India), University of Cambridge (UK),

Ohio State University (USA), McGill University (Canada), etc. Dr. Khush has been elected to world's most prestigious academies such as the US National Academy of Sciences, Royal Society of London, and Indian National Science Academy. He has received fellowships of several scientific societies. His alma mater, the University of California, Davis, awarded him its highest honor, a University Medal in 2018. Dr. Khush has supervised 49 M.Sc./ Ph.D. students, mentored 26 postdoctoral fellows, and trained numerous rice breeders. He has served as a member of editorial boards of more than 15 research journals. He has authored 3 books, edited 6 books, written 84 book chapters and 24 invited review articles, and published over 250 research papers in referred journals. Through a generous donation, Dr. Khush has established the "Dr. Gurdev Singh Khush Foundation for Advancement of Agricultural Sciences" at the Punjab Agricultural University, Ludhiana, 141004, India.

Foreword

I have known Dr. Gosal, the senior editor of this book, from my many visits to the reputable School of Biotechnology at the Punjab Agricultural University where he directed the laboratory and the school for decades. Experienced plant biotechnologists as they are, Dr. Gosal and Dr. Wani present an authoritative volume III book on *Biotechnologies of Crop Improvement*. This book covers 14 chapters. The first five chapters deal with the general subject matter of the science and practice of biotechnology for marker-assisted breeding for abiotic (salt tolerance, drought) and biotic (disease resistance) stresses and newly emerging field of genome editing. The last nine chapters deal on the application of various biotechnology tools for specific crops including rice, maize, wheat, sorghum, pearl millet, beans, groundnut, and oilseed. The crop-specific topics deal with myriad applications of biotechnology to wide hybrids, seed quality, genomic selection, abiotic stress, yield, and stress. Providing a broad coverage of topics and crops with useful applications of biotechnology, this book will be of interest to researchers as well as students and others with interest in crop improvement. I commend Dr. Gosal and Dr. Wani for the hard work in assembling this useful book on a relevant topic of wide interest and covering a diversity of crops that feed the world.

Bikram S. Gill
University Distinguished Professor and Director
Wheat Genetics Resource Center
Department of Plant Pathology
Kansas State University
Manhattan, KS 66506, USA

Preface

Conventional methods of plant breeding have been effectively used for the improvement of wide variety of crop plants. In the current scenario of global climate change, there is a need to integrate "genomics" in the plant breeding programs for rapid development and release of improved varieties. Since 1986, when Thomas H. Roderick, a geneticist, first coined the term "genomics" dealing with the structure and function of entire genome, there has been a huge expansion in this new subdiscipline of genetics. The term "genome" (derived from words genes and chromosomes) was first used by Hans Winkler (1920) to signify the complete set of chromosomes and their genes. In plants, initial genome mapping work in *Arabidopsis* and rice triggered research on other crops such as maize, wheat, rice, soybean, sorghum, pigeon pea, chickpea, brassica, and tomato. Genomics is finding huge applications in crop improvement to study gene number, gene mapping, gene sequencing, genome size, marker-assisted selection, QTL mapping, and transgenic breeding. Now the focus of plant breeding has increasingly switched from phenotype-based to genotype-based selection, "genomic selection" (GS). Several MAS strategies such as marker-assisted backcrossing, enrichment of favorable alleles in early generations, and selection for quantitative traits using markers at multiple loci have been developed. Whole genome and transcriptome sequencing bridges the gap between the genotype and phenotype. Furthermore, genome selection (GS) greatly speeds up the plant breeding programs. Genome editing with engineered nucleases such as zinc-finger nucleases, meganucleases, TALENs, and CRISPR can rapidly generate useful phenotypes/traits. Many crops like rice, maize, barley, and potato have been modified using these methods.

This book includes chapters prepared by specialists dealing with molecular breeding/genomics in relation to crop improvement. The first chapter deals with marker-assisted breeding for abiotic stress tolerance. The second chapter covers dynamics of salt tolerance, whereas the third chapter is devoted to marker-assisted breeding for disease resistance in crop plants. Further, a separate chapter deals with morphophysiological traits and molecular intricacies associated with tolerance to combine drought and pathogen stress. The fifth chapter exclusively deals with

genome editing for crop improvement. Subsequent chapters deal with molecular breeding/genomics of important field crops such as wheat, maize, basmati rice, groundnut, common bean, pearl millet, sorghum, chickpea, and oilseed brassicas.

This book provides state-of-the-art information on molecular breeding/genomics in relation to crop improvement. We earnestly feel that this book will be highly useful for students, research scholars, and scientists working in the area of crop improvement and biotechnology at universities, research institutes, R&Ds of agricultural MNCs for conducting research, and various funding agencies for planning future strategies.

We are highly grateful to all learned contributors, each of whom has attempted to update scientific information of their respective area and expertise and has kindly spared valuable time and knowledge.

We apologize wholeheartedly for any mistakes, omissions, or failure to acknowledge fully.

We would like to thank our families {Dr. Satwant Kaur Gosal (wife of SSG), Sana Ullah Wani, Taja Begum, Sheikh Shazia, Yasir Wani, and Muhammad Saad Wani (father, mother, wife, brother, and son of SHW)} for their continuous support and encouragement throughout the completion of this book.

We highly appreciate the all-round cooperation and support of Springer International Publishing AG, Cham, for their careful and speedy publication of this book.

Ludhiana, Punjab, India — Satbir Singh Gosal
Srinagar, Jammu and Kashmir, India — Shabir Hussain Wani

Contents

Contributors

P. K. Agrawal National Agricultural Science Fund, Indian Council of Agricultural Research, New Delhi, India

Parvaiz Ahmad Botany and Microbiology Department, College of Science, King Saud University, Riyadh, Saudi Arabia

Nudrat Aisha Akram Department of Botany, Government College University, Faisalabad, Pakistan

C. Bharadwaj Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India

P. K. Bhowmick Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India

Srikanth Bollam International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India

Nolan Bornowski Department of Plant, Soil and Microbial Sciences Michigan State University, East Lansing, MI, USA

Girish Chander International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India

Sushil Chaturvedi ICAR-Indian Institute of Pulses Research, Kanpur, Uttar Pradesh, India

Parveen Chhuneja School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, India

Mukesh Choudhary ICAR-Indian Institute of Maize Research, New Delhi, India

Paul Joseph Collins Plant, Soil and Microbial Science, Michigan State University, East Lansing, MI, USA

Santosh Deshpande International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India

R. K. Ellur Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India

S. Gopala Krishnan Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India

Satbir S. Gosal Punjab Agricultural University, Ludhiana, India

H. S. Gupta ICAR-Indian Agricultural Research Institute, New Delhi, India

Rajeev Gupta International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India

B. Haritha Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India

Vadivelmurugan Irulappan National Institute of Plant Genome Research, New Delhi, India

Suruchi Jindal School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, India

Amita Kaundal USDA-ARS, US Salinity Lab, Riverside, CA, USA

Maninder Kaur School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, India

Satinder Kaur School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, India

James D. Kelly Department of Plant, Soil and Microbial Sciences Michigan State University, East Lansing, MI, USA

Pardeep Kumar ICAR-Indian Institute of Maize Research, New Delhi, India

M. G. Mallikarjuna Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India

Pooja Manchanda School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, India

M. Nagarajan Rice Breeding and Genetic Research Centre, Division of Genetics, ICAR-Indian Agricultural Research Institute, Aduthurai, India

Manish K. Pandey International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India

B. S. Patil Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India

Vijayalakshmi Pujarula International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India

Kirandeep K. Romana International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India

Manish Roorkiwal Centre of Excellence in Genomics, ICRISAT, Patancheru, India

Supriya Sachdeva Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India

Devinder Sandhu USDA-ARS, US Salinity Lab, Riverside, CA, USA

Surinder K. Sandhu Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana, India

Muthappa Senthil-Kumar National Institute of Plant Genome Research, New Delhi, India

A. K. Singh Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India

Gurpreet Singh PAU-Regional Research Station, Faridkot, Punjab, India

Rajesh Kumar Singh Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India

Rakesh K. Srivastava International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India

Yadhu Suneja Department of Biochemistry, Punjab Agricultural University, Ludhiana, India

Challa Surekha Department of Biochemistry and Bioinformatics, Institute of Science, GITAM University, Visakhapatnam, AP, India

Rajeev Varshney Centre of Excellence in Genomics, ICRISAT, Patancheru, India

Rajeev K. Varshney International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India

K. K. Vinod Rice Breeding and Genetic Research Centre, Division of Genetics, ICAR-Indian Agricultural Research Institute, Aduthurai, India

Shabir H. Wani Mountain Research Centre for Field Crops, Khudwani, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar, India

Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, MI, USA

Zixiang Wen Plant, Soil and Microbial Science, Michigan State University, East Lansing, MI, USA

Shichen Zhang Agronomy Department, Kansas State University, Manhattan, KS, USA

About the Editors

Satbir Singh Gosal received his B.Sc. (Med.) from Panjab University, Chandigarh, India, and M.Sc. and Ph.D. (Plant Breeding) from Punjab Agricultural University, Ludhiana, India. He was awarded fellowships by the Royal Society London and the Rockefeller Foundation (USA) for his postdoctoral research at the University of Nottingham, England, and John Innes Centre, Norwich, England. Dr. Gosal has served in Punjab Agricultural University as Professor of Biotechnology, Director of School of Agricultural Biotechnology, and Additional Director of Research, and Director of Research. He has also served in FAO/IAEA, Vienna, Austria, and took tissue culture expert mission to Iraq in 1997. Dr. Gosal had rigorous training on "Biosafety of GM crops" from the Donald Danforth Plant Science Center, St. Louis, and APHIS, EPA (USDA), and USTDA, Washington, DC, USA. He has been an Honorary Member of the Board of Assessors, Australian Research Council, Canberra; President of the Punjab Academy of Sciences; elected member (Fellow) of the Plant Tissue Culture Association (India); and Fellow of Indian Society of Genetics and Plant Breeding. He is a recipient of Distinction Award from Society for the Promotion of Plant Science Research, Jaipur, India (2009), Fellow of the Punjab Academy of Sciences, and advisory member of several universities/institutes in the area of biotechnology. He served as a member of Review Committee on Genetic Manipulation (RCGM) for 3 years in the Department of Biotechnology (DBT), Government of India, New Delhi, and is a member of panel of experts in the area of Biotechnology for National Fund for Strategic Research of Indian Council of Agricultural Research, New Delhi. He has participated in more than 125 national/international conferences/meetings held in India, England, Scotland, Yugoslavia, Philippines, Indonesia, Thailand, the Netherlands, Malaysia, Singapore, Austria, Iraq, P R China, Australia, Mexico, Germany, and the USA. He has guided more than 75 (M.Sc. and Ph.D.) students for theses research on various aspects of plant tissue culture and plant transformation. He executed more than 20 externally funded research projects funded by Punjab State Government and various national and international organizations such as ICAR, DBT, DAC NATP, FAO/IAEA, and the Rockefeller Foundation, USA. He has more than 200 research

papers in refereed journals of high repute, 135 research papers in conference proceedings, several T.V./radio talks, and 30 book chapters. He has coauthored five laboratory manuals, one textbook, and two edited books.

Shabir Hussain Wani is Assistant Professor cum Scientist, Plant Breeding and Genetics, at the Mountain Research Centre for Field Crops, Khudwani, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar, Jammu and Kashmir, India, since May 2013. He received his B.Sc. in Agriculture from Dr. Bhimrao Ambedkar University, Agra, India, and M.Sc. and Ph.D. in Genetics and Plant Breeding from Central Agricultural University, Manipur, and Punjab Agricultural University, Ludhiana, India, respectively. His Ph.D. research fetched the first prize in north zone at national level competition in India. After obtaining his Ph.D., he worked as research associate in the Biotechnology Laboratory, ICAR-Central Institute of Temperate Horticulture, Rangreth, Srinagar, India, for 2 years, up to October 2011. In November 2011, he joined the Krishi Vigyan Kendra (Farm Science Centre) as program coordinator (i/c) at Senapati, Manipur, India. He teaches courses related to plant breeding, seed science and technology, and stress breeding. He has published more than 100 scientific papers in peer-reviewed journals and chapters in books of international and national repute. He has served as review editor of *Frontiers in Plant Sciences*, Switzerland, from 2015 to 2017. He is an editor of *SKUAST Journal of Research and LS: An International Journal of Life Sciences*. He has also edited ten books on current topics in crop improvement, published by reputed publishers including CRC press; Taylor and Francis Group, USA; and Springer. He is a fellow of the Linnean Society of London and Society for Plant Research, India. He received various awards including Young Scientist Award (Agriculture) 2015, Young Scientist Award 2016, and Young Achiever Award 2016 by various prestigious scientific societies. He has also worked as visiting scientist in the Department of Plant Soil and Microbial Sciences, Michigan State University, USA, during 2016–2017 under the Raman Fellowship for Post Doctoral Research program sponsored by the University Grants Commission, Government of India, New Delhi. He is a member of the Crop Science Society of America.

Chapter 1
Marker-Assisted Breeding for Abiotic Stress Tolerance in Crop Plants

Shabir H. Wani, Mukesh Choudhary, Pardeep Kumar, Nudrat Aisha Akram, Challa Surekha, Parvaiz Ahmad, and Satbir S. Gosal

Abstract The last few decades are evident of the economical uses of utilizing molecular markers of interested genes in plant breeding programs. The potential benefits of these traced markers of bona fide traits enhanced the feasibility of this marker-assisted selection (MAS). In traditional breeding programs, selection is carried out on morphological basis without knowledge of genetics. Regarding the recent yield issues, ecological problems, and enhancing populations, characters related to environmental stress tolerance, disease resistant, mineral and osmotic requirement, etc. should be the public attention. The molecular-assisted selection technology suggests a rapid progress in choosing stress-acclimated crop plants with expanding accuracy of selection. Molecular-aided selection is promising pyramid target traits in a single progeny more conveniently and precisely in few selected generations and little accidental harms. As it is a cost-effective and less time-consuming strategy, it can be suggested for long-term improvement in stress tolerance of economically important crops with some limitations.

S. H. Wani (✉)
Mountain Research Centre for Field Crops, Khudwani, Sher-e Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar, Jammu and Kashmir, India

Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, MI, USA

M. Choudhary · P. Kumar
ICAR-Indian Institute of Maize Research, New Delhi, India

N. A. Akram
Department of Botany, Government College University, Faisalabad, Pakistan

C. Surekha
Department of Biochemistry and Bioinformatics, Institute of Science, GITAM University, Visakhapatnam, AP, India

P. Ahmad
Botany and Microbiology Department, College of Science, King Saud University, Riyadh, Saudi Arabia

S. S. Gosal
Punjab Agricultural University, Ludhiana, India

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1.1 Introduction

Molecular markers are fragment of DNA associated to biological traits determined by allelic loci of genes and could be genetically transferred and can be handled as genetical tools or probes in order to keep mark of a gene, chromosome, tissue, or individual (Xu and Crouch 2008). Molecular markers are utilized in plant breeding and inherited programs. They can be divided into two classes, DNA markers and classical markers (Xu 2010). Based on various polymorphism-searching methods, DNA markers have been used in different systems (Collard et al. 2005), for example, single nucleotide polymorphisms (SNP), amplified fragment length polymorphisms (AFLP), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), and sequence-tagged sites (STS), etc. The classical markers comprised of biochemical markers, cytological markers, as well as morphological markers (Ashraf et al. 2012). The improvement in molecular-assisted breeding can solve the time limit problem of conventional breeding (Kage et al. 2016). Different molecular markers introduced in breeding programs since 1980 are AFLP, RFLP, and SSR markers (Kage et al. 2016). Successful application of MAS program in various crops to enhance the efficiency of conventional breeding was reported in rice (Jena and Mackill 2008), wheat (Miedaner and Korzun 2012), maize (Prasanna et al. 2010), and sorghum (Mohamed et al. 2014), etc.

Phenotypical neutral DNA markers are mostly used in breeding programs. Such markers are traced from vicinity of targeted genes. The disadvantage of selecting DNA markers is that their predictive value depends upon specific linkage phase which lies between target locus and marker (Lubberstedt et al. 1998). In individual mapping population, quantitative trait loci (QTL) are best selection because its subset would be polymorphic in each population (Lubberstedt et al. 2005). Any recombinant event may damage linkage between target gene locus and marker. Under such circumstances, flanking markers are used to avoid recombination, but there is fear of losing specified locus as double crossing over may take place upon introgression (Toojinda et al. 1998). Transferring lengthy chromosomal region with target gene may prove detrimental due to posed linkage penalties of undesired nontargeted genes. For example, one of the studies in wheat elite germplasm lines, the entry of Fusarium-resistant QLTs, possesses nontargeted undesirable genes which affect agronomic traits (McCartney et al. 2007). Likewise in rice the introgression of QLT caused unwanted plant height (Venuprasad et al. 2012).

Plant breeding plays potential role in increasing the crop yield production despite of facing multi challenges like abiotic and biotic stresses (Fig. 1.1) (Simova-Stoilova et al. 2016). Considering the current biological issues such as overpopulation, adverse agri-ecology, search of feasible traits like abiotic stress tolerance, water-use efficiency, and plant disease resistance/tolerance should be the major concerns (Slafer et al. 2005; Pandey et al. 2017). Molecular marker exhibited genetical differences among species (George et al. 2013). Mainly molecular markers do not specify the particular genes instead they act as chromosomal landmarks of genes to facilitate access of target gene of desirable traits (Collard et al. 2005; Singh and Singh 2015). These markers have no role in morphology of any trait because they are found in vicinity of target genes (O'Boyle et al. 2007; Jiang

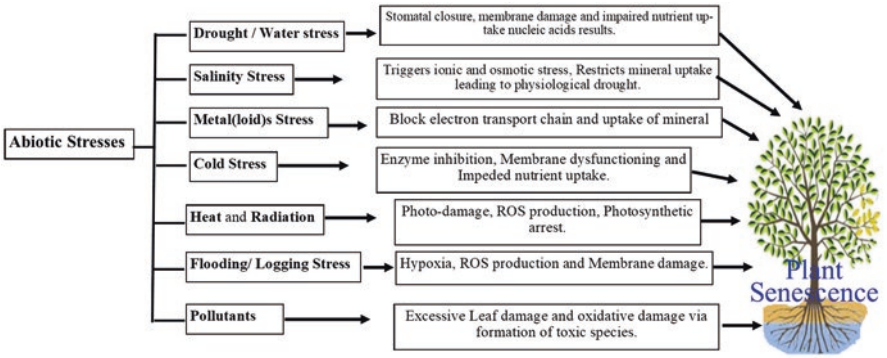

Fig. 1.1 Various types of abiotic stresses affecting plant growth and the complex mechanisms involved leading to plant senescence

2013). Molecular markers are specific sites and comprised of DNA nucleotide sequence and followed inheritance laws over generations and generations (Ruane and Sonnino 2007). The progress in molecular markers gave a significant advancement to eliminate the core restrictions of classical crop development programs in which breeding is processed on plant morphology, and this involves linkage drag. In this phenomenon, both desirable and undesirable genes are transferred due to strong interlinkage (Reddy 2017). For improving stress tolerance of cereal crops under stress adversaries, accelerated efforts have been done from last few decades. However, the issues such as accentuation of ecological stresses, moisture deficiency, and uncertain climatic changes caused major barriers for breeders to develop improved crop varieties (Lateef 2015). In order to combat these hardships, it is an utmost need of this era to utilize new technologies including molecular-assisted breeding to improve yield of cash crops. It provides an opportunity to select stress-tolerant crop plants with precise accuracy of selection (Kiriga et al. 2016). This chapter represents an overview about the basics of molecular mapping, molecular-assisted mapping, and its utilization in improving crop stress tolerance.

The molecular-assisted breeding programs have become two times faster than phenotypic-based breeding for different characteristics, i.e., abiotic and biotic stress tolerance, yield, as well as quality traits (Oliveira et al. 2008). The targeted alleles and their loci associated with abiotic stresses such as water logging, salinity, and desiccation have been traced and utilized in MAS (Baltazar et al. 2014; Kretzschmar et al. 2015; Gonzaga et al. 2016). In new challenging areas, the traditional, molecular-assisted, and DNA markers can be utilized for developing new multi-abiotic-resistant lines (Rumanti et al. 2016). In recent era, genetic information has been applied for salt and drought tolerance in different crops such as *Arabidopsis* (Nakashima et al. 2009), maize (Tollefson 2011), rice (Fukao and Xiong 2013), and *Brassica* (Zhang et al. 2014). Similarly, MAS has also developed waterlogged-tolerant lines in different crop plants (Devi et al. 2017). MAS has also improved agronomic characteristic of crop plants (Rozema and Flowers 2008). This chapter represents an overview about the basics of molecular mapping, molecular-assisted mapping, and its utilization in improving crop plants with special focus on stress tolerance.

1.2 Steps in Marker-Assisted Selection (MAS)

The molecular markers are used for the development of DNA marker maps. These marker maps are utilized to detect the presence of putative genes that affect the desired traits based on the degree of association between the marker and desired trait. MAS procedure involves five integral steps (Fig. 1.2) described below:

1.2.1 Selection of Plants with Desired Morphological Traits

Selecting suitable parents with desired traits is the fundamental basis of plant breeding. The plants are selected for superior agronomic traits, biotic and abiotic stress resistance, and other traits. Usually parents exhibiting contrasting traits or characters are selected because the main aim of this technique is to assemble major desirable gene combinations in the new crop variant. For selection of parents, germplasm screening is carried out followed by selecting parents with distinct DNA. Homozygous parents are considered for MAS. The size and composition of plant population

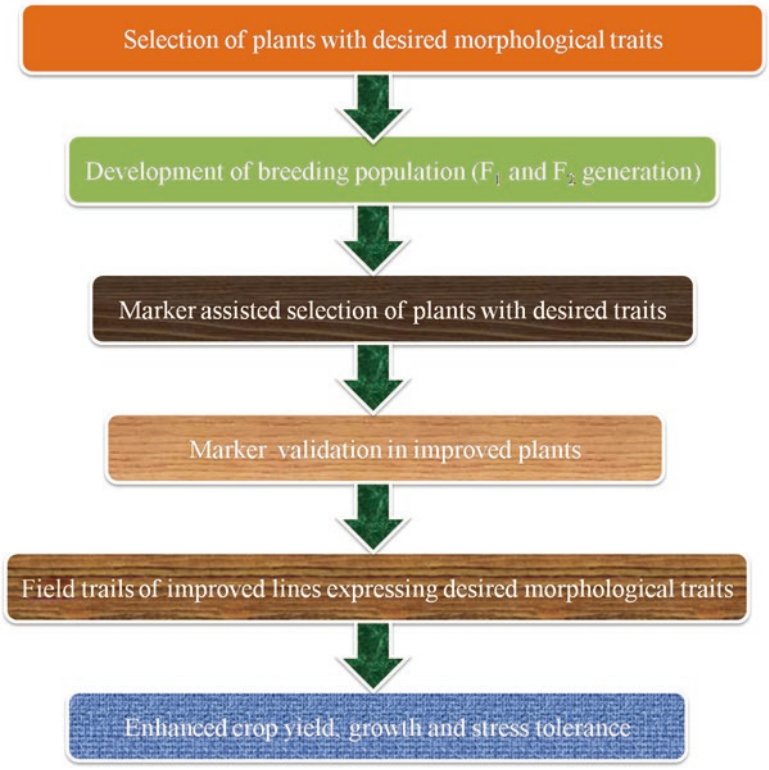

Fig. 1.2 Schematic representation of various steps involved marker assisted selection for abiotic stress tolerance in plants

determine the effectiveness of MAS. If the no. of genes segregating in a population is large, then more plant species need to be screened for identifying specific gene combinations (Witcombe and Virk 2001).

1.2.2 Development of Breeding Population

This is an essential step for marker-aided selection. After the parents are selected, they are crossed to obtain the F_1 generation. This is followed by screening the F_1 population to detect the presence of the marker alleles. The hybrids exhibiting the presence of markers are selected, while the remaining are eliminated. The next step involves developing the F_2 generation which is essential for analyzing the segregation pattern of RFLPs. Similar screening procedure is incorporated to distinguish the plants harboring the marker alleles.

1.2.3 Marker–Assisted Selection of Plants with Desired Traits

The next step of MAS involves isolating DNA from the above developed plant population. The DNA can be isolated from the seedling stage without waiting for the plants to grow and generate flowers for isolating their DNA. This considerably reduces the time for MAS procedure. The DNA is isolated from each plant of F_2 population using standard protocols. The isolated DNAs are subsequently digested with specific restriction enzymes to obtain various DNA fragments.

1.2.4 Marker Validation

The polymorphism or variation of the molecular marker (RFLP, RAPD, AFLP, SSR, SNP) between the parents and the presence of marker alleles in individuals of F_2 population is determined by using DNA probes complementary to the DNA marker sequences. In general phosphorous (^{32}P) is used for detecting the fragments bearing similarity. The probe hybridizes with specific segments which are complementary to it. This technique helps to identify the individuals carrying the marker alleles.

1.2.5 Correlation with Morphological Traits

The selected DNA marker is then correlated with morphological traits and confirms the process of indirect selection through DNA markers. Once the association of the DNA markers is established with morphological traits, MAS can be used for genetic crop improvement of agronomic traits. Field trials are conducted on the selected lines to evaluate the yield, quality, resistance, and other characters of interest.

1.3 MAB for Abiotic Stress Tolerance in Major Crop Plants

1.3.1 Rice

Drought stress is one of the major constraints in rice production and that too especially in rainfed conditions. Therefore, identification and introgression of consistent QTLs for drought resistance can prove to be an effective strategy to tackle the rice production in drought-prone areas. Although a number of QTLS have been identified in rice for drought resistance, the progress on marker-assisted backcrossing (MAB)-based introgression of the identified QTLS is limited (Table 1.1). Prince et al. (2015) conducted SSR-based QTL mapping on RIL population derived from a cross of IR20 and Nootripathu for physiological and yield traits under drought. Three QTLs mapped on chromosome 1 (*RM8085*), chromosome 4 (*I12S*), and chromosome 6 (*RM6836*) for physiological and yield traits can be effectively utilized for introgression into elite rice lines for stable yield production under drought stress-prone ecologies. In an another study, RILs and association mapping population were utilized for identification of QTLs for deep rooting as it is an important trait for imparting drought tolerance in rice and usually represented by ratio of deep rooting (RDR). SNP-based genotyping resulted into mapping of six QTLS for RDR on chromosomes 1, 2, 4, 7, and 10 (Lou et al. 2015). Sangodele et al. (2014) identified ten SSR genotyping-based QTLs for physiological, productivity, and related traits under drought using backcross inbred lines (BILs) derived from the cross of Swarna and WAB 450 and grown in a poly house in a randomized block design. The drought resistance imparted due to the positive alleles from WAB 450 can help in providing stability in grain yield under drought stress. Lang et al. (2013) worked on BC_2F_2 population derived from a cross of OM1490 and WAB880–1-38-18-20-P1- HB and identified four QTLS related to root length and root dry weight. The phenotypic variation explained by the QTLS for dry root weight lies in the range of 20.7% to 30.8%.

In the era of climate change, heat stress threatens the rice production globally. In this study, two different populations (biparental F_2 population and three-way F_2 population) derived from cross of heat-tolerant variety Giza178 × IR64 and IR64 × Milyang23 × Giza178, respectively. Results – four QTLs, namely, qHTSF1.2, qHTSF2.1, qHTSF3.1, and qHTSF4.1 and two QTLs, qHTSF6.1 and qHTSF11.2 – were identified in the biparental F_2 and three-way F_2 population, respectively, through linked SNP markers. The SNP markers can be further fine mapped to develop SNP chips for marker-assisted crop improvement (Ye et al. 2015). In a population of chromosome segment substitution lines derived from a cross of Sasanishiki (Japonica ssp. heat susceptible) and Habataki (Indica spp. heat tolerant), 11 QTLs were mapped through SSR markers on chromosomes 1, 2, 3, 4, 5, 7, 8, 10, and 11 for spikelet fertility, daily flowering time, and pollen shedding under heat stress. Three novel QTLs, namely, PSLht4.1, qPSLht7, and qPSLht10.2, were identified for the first time, and among them qPSLht4.1 was found to impart heat tolerance at varying temperature regimes and therefore can be successfully utilized for rice lines for better pollen shedding as well as pollen growth on stigma under heat stress (Zhao et al. 2016).

Table 1.1 List of few QTLs identified for abiotic stress tolerance in various crop plants

Crop	QTLs/Loci	Mapping Population	Cross(s)	Genotyping Markers	Environment	Chromosome	Stress	References
Rice	3 QTLS (physiological and yield traits)	RILs	IR20 × Nootripathu	SSRs	Field	1, 4, and 6	Drought	Prince et al. (2015)
Rice	6 QTLS (ratio of deep rooting)	RILS	Zhenshan97B × IRAT109	SNP	Field	1, 2, 4, 7, and 10	Drought	Lou et al. (2015)
Rice	4 QTLS (root length and root dry weight)	BC_2F_2	OM1490 × WAB880–1–38-18-20-P1- HB	SSRs	Greenhouse	2, 3, 4, 8, 9, 10, and 12	Drought	Lang et al. (2013)
Rice	15 QTLS (1000 grain weight, leaf temperature, relative water content, grain weight per plant, relative water content, productive tillers, grain number per plant, panicle weight, productive tillers, and spikelet fertility)	BIL	Swarna × WAB 450	SSR	Poly house	1, 2, 3, 7, 8, and 9	Drought	Sangodele et al. (2014)
Rice	11 QTLs (spikelet fertility, daily flowering time, and pollen shedding level)	CSSLs	Sasanishiki × Habataki	SSR	Field	2, 4, 3, 8, 10, 11, 5, and 7	Heat	Zhao et al. (2016)
Rice	8 QTLS (spikelet fertility)	Three-way cross	(IR64 × Milyang23) × Giza178	SNP	Net house	4	Heat	Ye et al. (2015)
Rice	5 QTLS (submergence tolerance beyond SUB1)	RILs	IR42 × FR13A	SSR	Greenhouse	1, 4, 8, 9, and 10	Water logging/ submergence	Gonzaga et al. (2016)
Rice	32 QTLS (shoot length, root length, and shoot fresh weight)	BILs	(Nipponbare×Kasalath) × Nipponbare	RFLPs	Glasshouse	1, 3, 4, 6, and 7	Water logging/ submergence	Manangkil et al. (2013)
Rice	4 QTLS (submergence)	$F_{2:3}$	IR72 × Madabaru	SNP	Net house	1, 2, 9, and 12	Water logging/ submergence	Septiningsih et al. (2012)
Rice	85 QTLs (shoot potassium concentration, sodium-potassium ratio, salt injury score, plant height, and shoot dry weight.)	RILs	Bengal × Pokkali	SNP	Greenhouse	1, 2, 3, 4, 6, 7, 8, 10, 11, and 12	Salinity	Leon et al. (2016)
Rice	16 QTLS (pollen fertility, Na^+ concentration, and Na/K ratio in the flag leaf)	F_2	Cheriviruppu × Pusa Basmati1	SSR	Net house	1,7,8, and 10	Salinity	Hossain et al. (2015)

(continued)

Table 1.1 (continued)

Crop	QTLs/Loci	Mapping Population	Cross(s)	Genotyping Markers	Environment	Chromosome	Stress	References
Rice	28 QTLs (different morphological and physiological traits)	BC_3DH	(Caiapo × O. glaberrima) × Caiapo	SSR	Growth chamber	5 and 10	Iron (Fe) toxicity	Dufey et al. (2015)
Rice	7 QTLs (leaf bronzing)	RILs	IR 29 × Pokkali	SSR and SNP	Hydroponics	1, 2, 4, 7, 12	Iron (Fe) toxicity	Wu et al. (2014)
Rice	3 QTLs (leaf bronzing)	BILs	Nipponbare × Kassalath	SSR and SNP	Hydroponics	1, 3,8	Iron (Fe) toxicity	Wu et al. (2014)
Rice	9 QTLs (culm length, shoot dry weight, and root dry weight)	CSSLs	Koshihikari × Kasalath	SSR	Greenhouse	3,8	Iron (Fe) toxicity	Fukuda et al. (2012)
Wheat	3 QTLs (yield and biomass)	NILs	Wild emmer wheat (*Triticum turgidum* ssp. *dicoccoides*) and durum (*T. turgidum* ssp. *durum*) and bread wheat (*T. aestivum*)	SNP	Net house	1BL, 2BS, and 7AS	Drought	Merchuk-Ovnat et al. (2016)
Wheat	4 QTLs (net photosynthesis, water content, and cell membrane stability)	F_2	Chakwal-86 × 6544–6	SSR	Hydroponics	2A	Drought	Malik et al. (2015)
Wheat	13 main QTLs (ABA content)	F_4	Yecora Rojo × Pavon 76	TRAP, SRAP, and SSR	Field	3B, 4A, and 5A	Drought	Barakat et al. (2015)
Wheat	22 QTLs (coleoptile length, seedling height, longest root length, root number, seedling fresh weight, stem and leaf fresh weight, root fresh weight, seedling dry weight, stem and leaf dry weight, root dry weight, root-to-shoot fresh weight ratio, and root-to-shoot dry weight ratio)	RILs	Weimai 8 × Luohan 2 Weimai 8 × Yannong 19	SSR, ISSR, STS, SRAP, and RAPD	Laboratory	1B, 2A, 2B, 3B, 4A, 5D, 6A, 6D, 7B, and 7D.	Drought	Zhang et al. (2013a)
Wheat	6 QTLs (seminal root angle and seminal root number)	DHs	SeriM82 × Hartog	DArT and SSR	Gel chambers	2A, 3D, 6A, 5D, 4A, 1B, 3A, 3B, and 6B	Drought	Christopher et al. (2013)
Wheat	20 major and minor QTLs (1000 grain weight, grain weight per spike, number of grains per spike, spike number per m^2, spike weight, spike harvest index, and harvest index)	F_3 and F_4	Oste-Gata ×Massara-1	SSR	Field	3B, 7B,1B, 2B, 1B, and 3B	Drought	Golabadi et al. (2011)

Wheat	37 QTLs (parameters of chlorophyll fluorescence kinetics)	DH	Hanxuan 10 × Lumai 14	AFLP and SSR	Growth chamber	1A, 1B, 2B, 4A, and 7D	Heat	Azam et al. (2015)
Wheat	5 QTLs (thylakoid membrane damage, plasma membrane damage, and SPAD chlorophyll content)	RILs	Ventnor × Karl 92	SNP	Greenhouse	6A, 7A, 1B, 2B, and 1D	Heat	Talukder et al. (2014)
Wheat	7 stable QTLs (grain yield, thousand grain weight, grain filling duration, and canopy temperature)	DH	Berkut× Krichauff	SSR	Field	1D, 6B, 2D, and 7A	Heat	Tiwari et al. (2013)
Wheat	3 QTLs (grain yield, thousand grain weight, grain filling duration, and canopy temperature)	RILs	NW1014 HUW468	SSR	Field	2B, 7B, and 7D	Heat	Paliwal et al. (2012)
Wheat	14 QTLs (three main spike yield components; kernel number, total kernel weight, and single kernel weight)	RILs	Halberd Karl 92	SSR	Greenhouse	1B, 2D, 3B, 4A, 5A, 5B, 6D, 7A, and 7B	Heat	Esten Mason et al. (2011)
Wheat	1 QTL (proportion of dead leaves)	Varieties	Durum wheat	SSR	Glasshouse	4B	Salinity	Turki et al. (2015)
Wheat	18 additive and 16 epistatic QTLs (the root, shoot and total dry weight, K^+, Na^+ concentration, and K^+/Na^+ ratio)	RILs	Chuan 35,050 × Shannong 483	SSR	Glasshouse	1A, 2A, 4B, 5D, 1B, 3A, 6D, 7B, 1D, 2B, 5A, 5B, 7A, 4A, 6A, and 6B	Salinity	Xu et al. (2013)
Wheat	36 QTLs and 10 QTLs (root dry weight index, shoot dry weight index, total dry weight index) respectively	RILs	W7984 × Opata85 SHWL1 × Chuanmai 32	DArT and SSR	Glasshouse	18 chromosome	Water logging	Yu and Chen (2013)
Wheat	2 QTLs (hematoxylin stain score and net root growth)	RILs	FSW × Wheaton	SSR	Greenhouse	4DL and 3BL	Aluminum (Al) toxicity	Dai et al. (2013)
Maize	169 QTLs (grain yield per plant, ear length, kernel number per row, ear weight, and hundred kernel weight)	NAM	11 biparental families (2000 RILs)	SNPs	Field	1, 3 and 10	Drought	Li et al. (2016)
Maize	203 QTLs (ASI, ears per plant, stay-green and plant-to-ear height ratio)	RILs $F_{2:3}$ $F_{2:3}$	CML444 × MALAWI CML440 × CML504 CML444 × CML441	SNPs and SSRs	Field	1, 3, 4, 5, 7, and 10	Drought	Almeida et al. (2014)

(continued)

Table 1.1 (continued)

Crop	QTLs/Loci	Mapping Population	Cross(s)	Genotyping Markers	Environment	Chromosome	Stress	References
Maize	45 QTLs (grain yield per plant and yield components)	$F_{2:3}$	B73 × DTP79	SSRs	Field	1, 2, 3, 4, 5, 6, 7, 8, and 10	Drought	Nikolic et al. (2013)
Maize	145 QTLs (grain yield, ASI), 7 mQTL for grain yield, and 1 mQTL for ASI	RILs $F_{2:3}$ $F_{2:3}$	CML444 × MALAWI CML440 × CML504 CML444 × CML441	SNPs	Field	1, 2, 3, 4, 5, 7, 8, and 10	Drought	Almeida et al. (2013)
Maize	64 QTLs (grain yield, number of kernels per row, number of rows per ear, ear length, ASI, visually scored drought score, relative water content, osmotic potential, and relative sugar content)	$F_{2:3}$	B73 × DTP79	RFLPs, SSRs, and AFLPs	Field	1, 2, 3, 4, 5, 7, 8, and 10	Drought	Nikolic et al. (2012)
Maize	43 QTLs (QTLs associated with grain yield, leaf width, plant height, ear height, leaf number, tassel branch number, and tassel length)	F_2	B73 × DTP79	RFLPs, SSRs, and AFLPs	Field	1, 2, 3, 4, 5, 6, 7, 8, and 10	Drought	Nikolic et al. (2011)
Maize	17 QTLS (leaf chlorophyll, plant senescence, electric root capacitance)	RILs	CML444 × SC-Malawi	SSRs	Field	1, 2, 4, 5, 6, and 10	Drought	Messmer et al. (2011)
Maize	25 QTLs (ASI, plant height, grain yield, ear height, and ear setting)	$F_{2:3}$	D5 × 7924	SSRs	Rain shelter	1, 2, 3, 4, 6, 8, 9, and 10	Drought	Zhu et al. (2011)
Maize	22 QTLs (sugar concentration, root density, root dry weight, total biomass, relative water content, and leaf abscisic acid content)	$F_{2:3}$	DTP79 × B73	RFLP	Greenhouse	1, 3, 5, 6, 7, and 9	Drought	Rahman et al. (2011)
Maize	6 QTLs (ph 6–1, rl1–2, sdw4–1, sdw7–1, tdw4–1, and tdw7–1)	$F_{2:3}$	HZ32 × K12	SSRs	Glasshouse	1, 4, 6, 7, and 9	Waterlogging	Osman et al. (2013)
Maize	18 QTLs (yield, brace roots, chlorophyll content, % stem, and root lodging)	RILs	CML311-2-1-3 × CAWL-46-3-1	SNP markers using KASP platform	Field	1, 2, 3, 4, 5, 7, 8, and 10	Waterlogging	Zaidi et al. (2015)
Maize	15 QTLs (seedling height, root length, shoot fresh weight, root fresh weight, shoot dry weight, and root dry weight)	BC_2F_2	K12 × HZ32	SSRs and SNPs	Greenhouse	5, 6, and 9	Waterlogging	Zhang et al. (2013b)

Maize	2 QTLs for aerenchyma formation (Qaer1.06–1.07 and Qaer7.01)	S1 and S2	*Z. nicaraguensis*	SSRs	Greenhouse	1 and 7	Waterlogging	Mano et al. (2012)
Maize	25 QTLs (total brace root tier number and effective brace root tier number)	RILs and immortalized F_2	Huangzao 4 × CML288	SSRs	Field	1, 2, 3, 5, 6, 7, 8, 9, and 10	Waterlogging	Ku et al. (2012)
Maize	27 QTLs (germination and early growth)	RILs	B73 × P39 and B73 × IL14 h	SNP	Field	1, 2, 3, 4, 5, 6, 7, 8, and 9	Cold	Allam et al. (2016)
Maize	6 QTLs (days to emergence)	Inbred populations	Two large panels of flint inbred lines	SNP	Growth chamber	3,4,5,7,10	Cold	Revilla et al. (2016)
Maize	15 QTLs (shoot length, root length, ratio of root length and shoot length shoot fresh weight, root fresh weight, plant fresh weight, plant dry weight, shoot dry weight, root dry weight, ratio root dry weight, and shoot dry weight)	F2:3	B73 and CZ-7	SSRs	Greenhouse	1, 2, 4, 5, 6, 7, 8, 9, and 10	Salinity	Hoque et al. (2015)
Common bean	14 QTLs (yield, yield per day and yield components, pod harvest index)	RILs	SEA5 × CAL96	SNP	Field	Pv01, Pv03, Pv04, Pv07, Pv08, and Pv09	Drought	Mukeshimana, et al. (2014)
Chickpea	48 QTLs (days to 50% flowering and maturity and days after sowing)	RILs	ICCV 2 × JG 11	SSR and SNP	Pots	CaLG05 and CaLG07	Salinity	Pushpavalli et al. (2015)
Chickpea	93 QTLs (plant height, days to flowering and days to maturity	RILs	ILC588 × ILC3279	SSR	Field	LG3 and LG4	Drought	Hamwieh et al. (2013)
	1 QTL (seed yield)	RILs	JG 62 × ICCV 2	SSR	Pots	LG3	Salinity	Vadez et al. (2012)
Cowpea	1 QTL (pod length and seed size)	F_2	*V. luteola* × *V. marina* subsp. *oblonga*	SSR	Hydroponics	LG1	Salinity	Chankaew et al. (2014)
Soybean	2 QTLs (root extension)	RILs	Young × PI 416937	RFLP and SSR	Hydroponics	Gm08 and Gm16	Al tolerance	Abdel-Haleem et al. (2014)
	4 QTLs (root length)	RILs	Essex × Forrest	BARC	Greenhouse	LGs B2, C2, F, and G	Al toxicity	Sharma et al. (2011)

(continued)

Table 1.1 (continued)

Crop	QTLs/Loci	Mapping Population	Cross(s)	Genotyping Markers	Environment	Chromosome	Stress	References
	1 QTL (salt tolerant)	RILs	PI 483463 × Hutcheson	SSR and SNP	Greenhouse	3	Salinity	Ha et al. (2013)
	7 QTLs (canopy wilting trait)	RILs	PI 416937 × Benning	SSR	Field	Gm12	Drought	Abdel-Haleem et al. (2012)
Pea	161 QTLs (height at harvest, number of basal branches per plant, pod number per plant, seed number per plant, seed number per pod, seed weight per plant, 1000-seed weight, straw dry weight per plant, biomass dry weight per plant, seed protein content, and harvest index)	RILs	JI1491 × Cameor	SSR and SNP	Field	1, 2, 3, 4, 5, 6, and 7	Frost	Klein et al. (2014)
	10 QTLs (relative water content in soil and leaves)	RILs	P665 × cv. Messire	SSR	Field	LGI, LGIII, and LGIV	Drought	Iglesias-García et al. (2015)

Submergence is a problem of serious concern in rice-growing ecologies particularly in South and Southeast Asia. Although *SUB1* gene has been utilized in rice to survive under complete submergence for a period of fortnight, then also novel QTLs are required to be identified for longer-term submergence. A cross between IR72 and Madabaru was made to develop $F_{2:3}$ population, and using SNP markers, four QTLs were identified on chromosomes 1, 2, 9, and 12. A major QTL found on chromosome 1 explained the phenotypic variance of 52.3%. Gene pyramiding can be utilized for combining this novel QTL with *SUB1* and thereby further improving the submergence tolerance in rice growing in flood-prone areas (Septiningsih et al. 2012). Manangkil et al. (2013) identified 32 QTLS for seedling vigor under submergence in backcross inbred lines derived from a cross of Nipponbare × Kasalath and Nipponbare. RFLP markers mapped 7, 11, and 14 QTLs for shoot length, root length, and shoot fresh weight on chromosomes 1, 3, 4, 6, and 7. Recombinant inbred lines (RILs) derived from a cross of IR42 and FR13A led to detection of five QTLs on chromosomes 1, 4, 8, 9, and 10. The novel QTLs have a tremendous potential to augment *SUB1* for better rice production under submergence conditions (Gonzaga et al. 2016).

Salinity is another important stress affecting the production of rice globally. Hossain et al. (2015) conducted QTL mapping in F_2 population derived from a cross of salinity-tolerant Cheriviruppu with sensitive cultivar Pusa Basmati 1 (PB1) using 131 SSR markers and mapped 16 QTLs for different traits such as pollen fertility, Na^+ concentration, and Na/K ratio on chromosomes 1, 7, 8, and 10. Similarly, another experiment resulted into identification of 16 major QTLs for various traits related to salinity tolerance such as shoot potassium concentration, sodium-potassium ratio, salt injury score, plant height, and shoot dry weight in RILs developed from a cross of Bengal with Pokkali. A total of 85 additive QTLs were mapped through 9303 SNPs on chromosomes 1, 2, 3, 4, 6, 7, 8, 10, 11, and 12 (Leon et al. 2016). These QTLs for salinity tolerance can be effectively utilized for marker-assisted breeding-based rice improvement for salinity tolerance.

Lowland rice is hampered by the problem of iron (Fe) toxicity owing to excess ferrous iron (Fe^{2+}) formation in reduced soils. African rice (*Oryza glaberrima*) is a potential source of genes for resistance to iron toxicity because of its adaptability to adverse soil conditions and higher rusticity. Therefore , SSR-based QTL mapping carried out in BC_3DH lines derived from the backcross of *O. sativa* (Caiapo)/*O. glaberrima* (MG12)//*O. sativa* (Caiapo) under Fe^{2+} condition in hydroponics resulted in identification of 28 QTLs for 11 morphological and physiological traits on chromosome 5 and 10 (Dufey et al. 2015). Similarly, Wu et al. (2014) identified seven QTLs for leaf bronzing score on chromosome 1, 2, 4, 7, and 12 in RIL population derived from a cross between IR29 and Pokkali.

1.3.2 Wheat

Moisture stress is a limiting factor in wheat production globally which can be addressed effectively through introgression of drought-tolerant QTLs in wheat cultivars. Merchuk-Ovnat et al. (2016) identified three QTLs for yield and biomass in RILs derived from a cross of wild emmer wheat (*Triticum turgidum* ssp. *dicoccoides*)

and durum (*T. turgidum* ssp. *durum*) and bread wheat (*T. aestivum*) on chromosomes 1BL, 2BS, and 7AS where wild emmer wheat acts as source of drought resistance. In another study on identification of QTLs for drought resistance in wheat, Barakat et al. (2015) identified 13 QTLs for abscisic acid content in F_4 population derived from a cross of drought-sensitive (Yecora Rojo) and drought-tolerant (Pavon 76) using different markers (SRAP, TRAP, and SSR). The QTLs mapped on chromosomes 3B, 4A, and 5A through linked markers (Barc164, Wmc96, and Trap9) can be used for breeding drought-tolerant cultivars. Similarly, QTL mapping conducted in F_2 population derived from cross of tolerant cultivar, Chakwal-86, with sensitive cultivar, 6544-6, using SSR markers mapped four QTLs for photosynthesis, cell membrane stability, and relative water content on chromosome 2A (Malik et al. 2015). Zhang et al. (2013a) identified 22 QTLs on chromosomes 1B, 2A, 2B, 3B, 4A, 5D, 6A, 6D, 7B, and 7D for different traits like coleoptile length, seedling height, longest root length, root number, seedling fresh weight, stem and leaf fresh weight, root fresh weight, seedling dry weight, stem and leaf dry weight, root dry weight, root-to-shoot fresh weight ratio, and root-to-shoot dry weight ratio in two RIL populations derived from Weimai 8 × Luohan 2 and Weimai 8 × Yannong 19, respectively. Six QTLs found to be major or main effect QTLs can be utilized for drought improvement in wheat breeding program. Root architectural traits can play an important role in imparting resistance to drought in wheat. Four QTLs and two QTLs for seminal root angle and seminal root number, respectively, were mapped through DArT and SSR markers in a doubled haploid population derived from a cross of SeriM82 and Hartog. Four QTLs for seminal root angle were located on chromosomes 2A, 3D, 6A, and 6B, while for two QTLs for seminal root number on 4A and 6A (Christopher et al. 2013).

Wheat-growing ecologies are severely affected due to heat (high temperature) stress during grain filling period. In present scenario terminal heat stress is a major concern for wheat production globally. Parameters of chlorophyll fluorescence kinetics (PCFKs) can be utilized for identification and understanding of heat stress-tolerant cultivars. Therefore, QTL mapping was done in a DH population derived from a cross of Chinese cultivars, Hanxuan 10 and Lumai 14, using SSR and AFLP markers under controlled conditions. In the study, a total of seven QTLs were mapped on chromosomes 1A, 1B, 2B, 4A, and 7D for traits related to PCFKs such as initial fluorescence, maximum fluorescence, variable fluorescence, and maximum quantum efficiency of photosystem II. These identified QTLs can be effectively utilized for further deep understanding of genetic basis of chlorophyll fluorescence kinetics (Azam et al. 2015). Similarly, Talukder et al. (2014) targeted mapping of QTLs for thylakoid membrane damage (TMD), plasma membrane damage (PMD), and SPAD chlorophyll content (SCC) in RIL population developed from a cross of Ventnor and Karl 92. This is the first study on the development of genotype by sequencing SNP (GBS-SNP) marker-based linkage map in wheat. There are a total of five QTLs for TMD, PMD, and SCC on chromosomes 1B, 1D, 1B, 2B, 6A, and 7A with a maximum phenotypic variance of 33.5% for PMD. Tiwari et al. (2013) conducted SSR-based QTL mapping in DH population derived from a cross of Berkutwith and Krichauff and identified a total of seven stable QTLs on chromosomes 1D, 6B, 2D, and 7A. Three, two, and one QTLs were identified for grain filling duration, thousand grain weight, grain yield, and canopy temperature.

Salinity is a major concern for wheat production globally. Improved wheat cultivars with salinity tolerance can be developed through identification and introgression of QTLs imparting tolerance to salinity. Proportions of dead leaves (%DL) are an important parameter for identification of tolerant cultivars at early vegetative stage. SSR-based association studies carried out in 119 wheat varieties led to identification of a single QTL for %DL on chromosome 4B (Turki et al. 2015). Xu et al. (2013) identified a total of 18 additive and 16 epistatic QTLs in a RIL population comprising of 131 individuals, derived from a cross of Chuan 35,050 and Shannong 483. QTLs for shoot K^+/Na^+ concentration and for total dry weight mapped on chromosome 5A and chromosome 4B, respectively.

Globally, irrigated and high rainfall-prone wheat ecologies are severely affected by water logging. There have been very limited studies on identification of QTLs for waterlogging tolerance in wheat. Two independent RIL populations, International Triticeae Mapping Initiative (ITMI) population "W7984/Opata85" and "SHWL1 × Chuanmai 32" (SC) population, were mapped for root dry weight index, shoot dry weight index, and total dry weight index. DArT and SSR-based mapping led to identification of 36 and 10 QTLs in and SC population, respectively. These QTLs can be effectively utilized for development of waterlogging-tolerant varieties in wheat breeding program (Yu and Chen 2013).

Acidic soils tend to limit the wheat production globally due to aluminum (Al) toxicity, and development of Al-tolerant cultivars can effectively address the problem of wheat production in acidic soils. A RIL mapping population developed using Al-tolerant Chinese landrace FSW and Al-sensitive US spring wheat cultivar Wheaton was validated for previously identified QTL FSW. Out of three previously identified QTL for net root growth during Al stress, two QTLs were validated through earlier reported SSRs to be present on chromosome 4DL and 3BL. The linked SSR markers can be effectively utilized for development of AL-tolerant cultivars through MAS (Dai et al. 2013).

1.3.3 Maize

Drought is also a major abiotic constraint in maize that causes drastic yield reduction and economic losses in all over the world. Several researchers identified a large number of QTLs using different mapping population with different molecular markers, and these QTLs can address the drought problem effectively. Almeida et al. (2013) evaluated three tropical biparental populations under water stress (WS) and well-watered (WW) regimes to identify genomic regions responsible for grain yield (GY) and anthesis-silking interval (ASI) and identified a total of 83 and 62 QTL through individual environment analyses for GY and ASI, respectively. Six constitutively expressed meta-QTLs mapped on chromosomes 1, 4, 5, and 10 for GY. One mQTL on chromosome 7 for GY and one on chromosome 3 for ASI were found to be "adaptive" to WS conditions. In another study, Almeida et al. (2014) identified a total of 203 QTLs for different drought-related morphophysiological traits using biparental population, and a clusters of QTL were observed on chromosomes 1, 3,

4, 5, 7, and 10. Li et al. (2016) evaluated 5000 inbred lines from 30 joint linkage association mapping population using 365 SNPs for genome-wide association, and these SNPs associated with drought-related traits, located in 354 candidate genes. Fifty-two of these genes showed significant differential expression in the inbred line B73 under the well-watered and water-stressed conditions (Li et al. 2016).

Waterlogging is an important abiotic stress that leads to significant yield losses in high rainfall-prone maize-growing ecologies. The most sustainable and viable approach to tackle the waterlogging problem is through MAS-based incorporation of QTLs for waterlogging tolerance in cultivars. Zaidi et al. (2015) assessed the genetic variation in a population of recombinant inbred lines (RILs) derived from crossing a waterlogging-tolerant line (CAWL-46-3-1) to an elite but sensitive line (CML311-2-1-3) and observed significant range of variation for grain yield under waterlogging stress along with a number of other secondary traits such as brace roots (BR), chlorophyll content (SPAD), % stem, and root lodging (SandRL) among the RILs. Genotyping with 331 polymorphic single SNP markers using KASP (Kompetitive Allele Specific PCR) platform revealed a total of 18 QTLs on chromosomes 1, 2, 3, 4, 5, 7, 8, and 10.

Low temperature or cold is another type of abiotic stress that hampers the growth and yield of maize. Allam et al. (2016) developed two independent RIL populations from the crosses of B73 × P39 and B73 × IL14h and identified a total of 27 QTLs for germination and early growth under field condition. SNP genotyping mapped the QTLs on chromosomes 1, 2, 3, 4, 5, 6, 7, 8, and 9. Revilla et al. (2016) conducted a genome-wide association analysis in temperate maize inbred lines for pyramiding of cold tolerance genes and recorded traits for days from sowing to emergence and relative chlorophyll content using two panels of 306 dent and 292 European flint maize inbred lines. A total of 47 flint inbreds and 4 dent inbreds harbored the favorable alleles for 6 significant QTLs and 3 main effect QTLs, respectively, under cold conditions. These findings for cold tolerance will open new avenues for the genetic improvement of maize genotypes through marker-assisted selection or genome-wide selection.

Salinity also affects the maize production in the ecologies with higher proportion of saline soil. Different traits related to salt tolerance such as shoot length, root length, ratio of root length, and shoot length shoot fresh weight, root fresh weight, plant fresh weight, plant dry weight, shoot dry weight, root dry weight, ratio root dry weight, and shoot dry weight were targeted for mapping the QTLs in $F_{2:3}$ population developed from a cross between B73 and CZ-7 under greenhouse. SSR genotyping mapped 15 QTLs for target traits on chromosomes 1, 2, 4, 5, 6, 7, 8, 9, and 10 (Hoque et al. 2015).

1.3.4 Chickpea

Chickpea contributes a greater proportion to total pulse production, but it is vulnerable to low moisture in drought-prone areas**.** SSR-based QTL mapping conducted in a RIL population comprising of 181 individuals, derived from a cross of ILC588

and ILC3279, identified 93 QTLs for drought resistance score, plant height, days to flowering, and days to maturity along with seven yield-related traits such as grain yield, biological yield, harvest index, the number of pods/3 plants, percentage of empty pods, 100-seed weight, and seed number/3 plants. The QTLs mapped on LG3 and LG4 can play an immense role in chickpea molecular breeding programs for drought (Hamwieh et al. 2013).

In addition to drought, salinity is also an important abiotic stress hampering the chickpea production globally. Pushpavalli et al. (2015) identified 48 QTLs for days to 50% flowering and maturity and days after sowing in RILs derived from cross of ICCV 2 and JG 11. Twenty-eight SSRs and SNPs mapped the QTLs for salinity tolerance-related traits on linkage group 5 and 7.

1.3.5 Common Bean

Drought stress is a serious concern for common bean (*Phaseolus vulgaris* L.) production globally. A total of 14 QTLs were detected for traits such as number of days to flower, maturity, and seed fill; harvest index and pod harvest index; and yield and yield components including number of pods per plant, seeds per pod, 100-seed weight, and seed yield per day in RIL population derived from a cross of drought tolerant line SEA5 and CAL96 cultivar under. QTLs mapped under drought stress with the help of SNP markers on chromosomes 1, 3, 4, 7, 8, and 9 can be used for development of drought-tolerant cultivars in common bean molecular breeding program (Mukeshimana et al. 2014).

1.3.6 Soybean Al Tolerance

Soybean [*Glycine max* (L.) Merr.] serving as an important source of protein and oil is highly sensitive to aluminum (Al) toxicity existing in acidic soils globally. Therefore, breeding for Al-tolerant cultivars can provide a sustainable solution for Al toxicity in soybean. Root extension is an important trait that imparts tolerance to AL-toxicity under acidic soils. Two QTLs for root extension mapped on linkage group 8 and 16 in RILs derived from a cross of young and PI 416937 and evaluated under Al stress conditions (Abdel-Haleem et al. 2014).

Salt tolerance is another abiotic stress that hampers the soybean production in a significant manner. Ha et al. (2013) identified a single QTL for salt tolerance in RIL population derived from a cross of PI 483463 and Hutcheson. SSR- and SNP-based genotyping mapped the QTL for leaf scorching between SSR03_1335 and SSR03_1359 on chromosome 3. The linked markers can be effectively utilized for identification of salt-tolerant cultivars possessing desirable QTL and thereby enhancing the pace of soybean molecular breeding program for salt tolerance.

1.3.7 Pea

Winter pea crop production is hampered by the problem of frost in temperate regions. Therefore, there is a requirement of identification of genomic regions that can contribute to frost-tolerant cultivar development in winter pea. A total of 161 QTLs have been identified in a RIL population derived from cross of JI1491 and Came or for different traits such as height at harvest, number of basal branches per plant, pod number per plant, seed number per plant, seed number per pod, seed weight per plant, 1000-seed weight, straw dry weight per plant, biomass dry weight per plant, seed protein content, and harvest index under six environments. The mapping carried out with the help of SSR and SNP markers located the QTLs on seven linkage groups (Klein et al. 2014). Pea is an important legume crop whose production is affected by low moisture in drought-prone ecologies**.** Iglesias-García et al. (2015) identified ten QTLs (relative water content in soil and leaves) in RILs derived from a cross of P665 and cv. Messire using SSR markers. The QTLS located on linkage groups 1, 3, and 4 were found to be linked to markers such as A6, AA175, AC74, AD57, AB141, AB64, Psblox2, PsAAP2_SNP4, and DipeptIV_SNP1, and thereby these markers can aid for selection of drought-tolerant genotypes possessing desired QTLs.

1.4 Conclusion

Plant breeding coupled with latest agricultural technology has made significant progress in crop yield and quality improvement, and it is essential that this continue in the same way considering the population explosion. As discussed above MAS holds great promise for plant breeders to maintain sustainable crop yield. MAS has been successfully used for development of drought-tolerant rice (Jongdee et al. 2002), wheat (Mathews et al. 2008), and barley (Forster et al. 2000). Moreover MAS is highly useful to identifying QTLs associated with conferring abiotic stress tolerance. The only drawback of MAS is its high cost, in which, if regulated, MAS can be widely used for the development of crops bearing superior traits and qualities.

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Chapter 2
Dynamics of Salt Tolerance: Molecular Perspectives

Devinder Sandhu and Amita Kaundal

Abstract Plant being sessile has to face many environmental stresses and develop physiological and biochemical mechanisms to withstand those stresses. Salinity is one of the major stresses that affects the plant growth and imposes direct impact on productivity and yield. The lack of good-quality irrigation water is forcing farmers to use alternative or degraded waters of irrigation throughout the world. One of the major considerations for using alternative or degraded water is often its high salinity. Salinity affects various physiological and biochemical mechanisms in plants, including germination and growth, photosynthesis, and plant water relations. Plants, when exposed to external stresses, develop mechanisms and responses to defend themselves. Salt tolerance is regulated by a complex network of different component traits. With the availability of the genome sequences of the most crop species, it became feasible to develop the links between physiological performance and underlying biological mechanisms involved in salinity stress. This article describes the effect of salinity on germination, growth, photosynthesis and plant water relations, and the mechanism plant adopts to protect itself, such as ion exclusion from roots, sequestering ions into vacuole, and high tolerance to ion toxicity. Recent advances in research and technology may play critical role in developing new salt-tolerant cultivars that are vigorous and high yielding.

Keywords Salt tolerance · Salt stress · Ion homeostasis · Ion content · Ion transporters · Na^+/H^+ antiporters · Vacuolar compartmentalization

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

D. Sandhu (✉) · A. Kaundal
USDA-ARS, US Salinity Lab, Riverside, CA, USA
e-mail: Devinder.Sandhu@ARS.USDA.GOV

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2.1 Introduction

One of the biggest challenges farmers throughout the globe are facing is the availability of good-quality water for agriculture. Reduced availability of water and increasing demand of water by urban and industrial sectors due to industrialization are enforcing the use of alternative or degraded water for agriculture. One big consideration about using alternative or degraded water is its salt concentration. Salinity is a common problem throughout the world and is among one of the most important abiotic stresses faced by plants. About 20% of the irrigated agricultural land worldwide faces some level of salinity (Flowers and Yeo 1995). With the increasing scarcity of irrigation water, salinity problem is further pronounced. Salinity research is expected to widen in future years. There are many factors that affect soil salinity such as low precipitation, higher surface evaporation, low-quality irrigation, intensive farming, and poor drainage (Tanji 1990; Szabolcs 1994). About 10 million hectares of the land has been estimated to be destroyed by soil salinity each year (Pimentel et al. 2004). It is estimated that 50% of arable land will be affected by salinity by 2050 (Bartels and Sunkar 2005). On one hand, the food demand is growing with the increasing human population; on the other hand, due to increasing soil salinity, the availability of farming land is declining. Thus there is greater need to develop more sustainable crops to increase the productivity by utilizing saline water for irrigation.

Salt stress affects growth and development adversely by inhibiting many metabolic processes and cell expansion and by triggering programmed cell death (Huh et al. 2002). During initial phases of salinity stress, osmotic stress is predominant leading to increased transpiration and loss of absorption capacity of roots (Munns and Tester 2008). Osmotic stress is accompanied by several biochemical and physiological changes including disintegration of membranes, ion imbalance in the cells, decrease photosynthetic activity, and reduced stomatal conductance (Munns 2005; Munns and Tester 2008; Gupta and Huang 2014). Osmotic stress is also an initial step during drought. In response to osmotic stress, plants adjust osmotic pressure inside the cells, which normally happens within the first few hours following the salt stress. Gradually, concentrations of Na^+ and Cl^- increase inside cell cytoplasm resulting into ionic stress(Munns and Tester 2008). Although, ionic stress develops gradually, as it takes time for Na^+ or/and Cl^- concentration to reach the toxic levels, it becomes much more important as compared to the osmotic stress. High concentration of Na^+ not only causes ion imbalance, but it also interferes with K^+ absorption by roots leading to K^+ deficiency, which in turn results in reduced productivity (James et al. 2011).

Most salinity studies conducted in plants do not differentiate between salt stress and salt shock (Shavrukov 2013). When plant is exposed to a single application of high concentration of salt, it leads to plasmolysis resulting into an osmotic shock. Plant tries to cope with the osmotic shock by maintaining ion homeostasis. However, excessive salt concentration in the root cells causes leakage of salt solution in the apoplastic space, which quickly travels to the shoots without much control or regulation. This process leads to early ionic stress in the shoots causing overlap between gene expression relating to osmotic and ionic phases. These experiments identify very few genes associated with ionic phase. On the other hand, gradual increase in

salt concentrations results in salt stress, where there is not much overlap between osmotic and ionic responses, and these experiments are able to identify a large number of genes associated with ionic stress (Shavrukov 2013). In response to salt shock, several genes involved in cell turgor maintenance, accumulation of osmolytes, and water balance are induced within the first few hours of the salt treatment (Kawasaki et al. 2001). Several other genes involved in defense-related functions were upregulated in the first 24 hours, likely due to restoration of damaged components of the cell due to osmotic shock (Kawasaki et al. 2001). However, genes involved in ionic response were induced only after a week of salt treatment. In this study, many important genes including ion transporters were not upregulated suggesting that osmotic shock can alter the expression pattern of some stress-related genes which can lead to wrong inference (Shavrukov 2013). The comparison of expression of four superoxide dismutase (SOD) genes in one-step application and stepwise application of NaCl revealed that all four genes were highly upregulated over control in one-step application, whereas in stepwise increase of NaCl, none of the four genes were induced over control (Rubio et al. 2009). These observations suggested that these SOD genes are involved in osmotic shock rather than salt stress itself (Rubio et al. 2009). In field conditions, osmotic shock almost never happens. During spring, salt present on the soil surface moves to subsurface layers, and during summer salt gradually moves back to the upper layers slowly increasing the salt content in the root zone.

Salt tolerance is the ability of plant to survive under higher concentration of soluble salt in their rhizosphere. Salt tolerance is normally measured in terms of sustaining plant vigor and yield under saline conditions as compared to the controlled conditions. There are considerable differences in crop plants for salt tolerance. Barley and alfalfa are considered as moderately tolerant to salinity; on the other hand, *Arabidopsis*, strawberry, and avocadoes are very sensitive (Munns and Tester 2008; Suarez and Grieve 2013; Mauk et al. 2017; Sandhu et al. 2017). For the crops that are sensitive to salt, the specific ion toxicities are the primary cause for the salt stress as in these crops, the salinity damage occurs even in conditions when osmotic pressure is very low (Suarez and Grieve 2013).

In order to solve the complex puzzle of salinity, it is important to understand the genetic determinants that regulate biochemical and physiological responses and manipulate those to developing genetic material tolerant to salinity. The focus of this book chapter is to discuss recent advances in identifying genetic components of the salt tolerance mechanism and understanding their links with the biochemical and physiological responses.

2.2 Effect of Salinity on Plants

The salt stress affects the growth of a plant by limiting germination, leaf expansion, root/shoot growth, photosynthesis, stomatal conductance, and nutrient uptake (Chartzoulakis and Klapaki 2000; Wang and Nii 2000; Stępień and Kłbus 2006; Machado and Serralheiro 2017). All these factors ultimately affect the biomass and yield of the plant.

2.2.1 Germination and Growth

The high salt concentrations affect the plant growth and germination drastically. Salt inhibits the plant growth mainly due to two reasons. Firstly, salt in soil reduces the uptake of water by roots and secondly, salt enters the plant system and accumulates in the cell cytoplasm of different plant tissues, leading to ion toxicity (Munns 1993; Munns and Tester 2008). However, in the plant species that are highly sensitive to salinity, the two-phase effect of salinity is not visible (Munns and Tester 2008).

Salinity affects the plant throughout its life, though this sensitivity varies from one developmental stage to the other (Läuchli and Grattan 2007). Plants are generally tolerant to salt at germination but are sensitive to emergence and early vegetative growth (Lauchli and Epstein 1990; Maas and Grattan 1999). However, as a plant grows and matures, it becomes more tolerant to salt stress. At germination and emergence stage, the salt stress affects the survival rate, but at later stages, salt stress reduces the growth and yield parameters (Machado and Serralheiro 2017).

Seed germination is an important part of the life cycle of a plant. Though the germination stage is considered as the salt-tolerant stage, still the percentage of seed germination, the speed of germination, and the rate of emergence in various crops are decreased with increase in salinity (Carpici et al. 2009; Kaveh et al. 2011; Xu et al. 2011). The possible reason for this reduction is decreased absorption of water by the seed. Saline water has lower osmotic potential that results in reduced imbibition in seeds (Carpici et al. 2009). In addition, salt toxicity disturbs hormonal balance and affects activities of enzymes involved in nucleic acid and protein metabolism (Khan et al. 1994; Dantas et al. 2007).

Germination is known to be more tolerant than growth in most species. In chickpea, some genotypes that were not able to tolerate even 100 mM NaCl during growth could tolerate more than 300 mM NaCl during germination (Flowers et al. 2010). Similarly, seedling germination was significantly reduced at 100 mM of NaCl treatment, while seedling growth expressed as height, leaf area, and dry weight was reduced when salinity exceeded 10 mM NaCl in two greenhouse-grown bell pepper hybrids (Chartzoulakis and Klapaki 2000). On the similar lines, decrease was observed in root length, shoot length, and fresh biomass with increase in salt concentration in black gram (*Phaseolus mungo* L.) (Dash and Panda 2001).

At lower salt concentrations, some crops show improved germination rates. In quinoa, lower concentrations of different salts and sea water dilutions led to increase in germination rate as compared to control, which was attributed to activation of an effective antioxidant mechanism that resulted in improved performance under salt stress (Panuccio et al. 2014). In *Vicia faba*, although at high NaCl concentrations, there was a decrease in plant height, but at low and medium concentrations, increase in plant height was reported (Qados and Amira 2011). Authors attributed improved performance under low and medium salinity levels due to the osmotic adjustment activity leading to improved growth. Similarly, the increases in fresh weight were reported in other crops such as lettuce, cowpea, and pak choi upon low and medium salt treatments (Andriolo et al. 2005; Dantas et al. 2005; Memon et al. 2010). Crops such as spinach and sugar beet are known to utilize both

Na^+ and K^+ for growth and development and hence perform better under moderate levels of salinity (Kronzucker et al. 2013). The enhanced performance under salinity may be due to the use of Na^+ as nutrient by these plants when K^+ is deficient in the growth medium (Kronzucker et al. 2013).

Increased salt concentration in plant tissues can affect enzyme activities and interfere with metabolic and physiological processes. During germination, seeds absorb water that activates metabolic functions needed for germination. Seed porosity is an important parameter that helps in hydration and imbibition of seed during germination. Pectin is an important constituent of cell wall and commonly found in highly methylesterified form and regulates cell wall properties such as elasticity, ion binding, hydration, and porosity (Yan et al. 2018). Knockdown mutants for *pectin methylesterase 31* (*PME31*) resulted in increased sensitivity to salt during germination and led to downregulation of stress-related genes *DREB2A*, *RD29A*, *RD29B*, and *RD26* (Yan et al. 2018). Seeds liberate lots of energy during germination for the growth of the embryo. The increased levels of acid phosphatases were associated with increased levels of phosphate and energy liberation in seeds during germination (Nasri et al. 2016). Some *Arabidopsis* accessions that showed poor germination in 50 mM concentration of NaCl had diminished acid phosphatase activity; on the contrary the accession that showed 100% germination had increased acid phosphatase activity, suggesting that phosphatase may be critical for germination under salt stress conditions (Nasri et al. 2016). As the seeds germinate, endosperm cells enlarge and expand and cell walls in endosperm break. Expansin gene, *AtEXP2*, which is constitutively expressed during seed germination, is believed to be involved in breaking the cell wall in endosperm to make space for radicle emergence (Myburg et al. 2014). AtEXP2 has been shown to be involved in GA-mediated seed germination and provides enhanced tolerance to salt and osmotic stresses (Yan et al. 2014). Similarly, several genes have been characterized that play important role in growth during salinity stress. Transgenic tobacco plants containing the grape *Vv-α-gal/SIP* gene, involved in carbohydrate metabolism, showed enhanced salt tolerance as compared to wild-type (Daldoul et al. 2018). Salt hypersensitive mutant 9 (SAHY9/APUM23 protein) that plays an important role in the regulation of ribosome biogenesis is shown to be involved in growth during salt stress, and the salt hypersensitivity of the mutant is regulated through abscisic acid pathway (Huang et al. 2018). Identifying additional players involved in seed germination and growth under salinity stress may help in understanding metabolic control of salt tolerance in plants.

2.2.2 *Effect of Salinity on Photosynthesis and Photosynthetic Pigments*

Photosynthesis is a vital process needed by plants to convert solar energy into chemical energy. The decrease in photosynthesis rate under saline conditions is mainly attributable to reduction of water potential of a plant (Behboudian et al. 1986). Due to reduced water potential under saline condition, plant tries to retain water by closing stomata. Though this helps plant in controlling loss of water through

transpiration, closing stomata also prevents carbon dioxide intake and significantly affects photosynthetic efficiency (Behboudian et al. 1986). The stomatal conductance decreases considerably during salt stress, limiting the photosynthetic capacity of the plant (Chartzoulakis and Klapaki 2000; Meloni et al. 2003). The other factor affecting photosynthesis is accumulation of Na^+ in older leaves. In plants grown under saline condition, the net photosynthesis decreases in older leaves where Na^+ accumulates, although at whole plant level, there is no significant decrease (Yeo et al. 1985). The net photosynthesis is inversely related to Na^+ accumulation and decreases linearly with increase in Na^+ concentration in the leaves (Yeo et al. 1985). The same trend is observed for Na^+ accumulation and the transpiration rate (Yeo et al. 1985).

Salinity decreases the total chlorophyll and carotenoid contents in general (Munns and Tester 2008; Sandhu et al. 2017). In rice, 200 mM NaCl treatment for 14 days resulted in 41% and 33% reduction in the chlorophyll *a* and *b* contents in the leaves, respectively (Amirjani 2011). The total starch content decreased in the stressed seedlings, but the total reducing and nonreducing sugar contents increased (Amirjani 2011). The rate of net photosynthesis was also reduced in salt-stressed seedlings leading to overall growth reduction (Amirjani 2011). Following the similar pattern as rice, chlorophyll *a* in cucumber was affected to a lesser extent by NaCl as compared to chlorophyll *b* (Stępień and Kłbus 2006; Amirjani 2011). However, the salt stress reduced photosynthesis by both stomata closure and non-stomatal factors (Stępień and Kłbus 2006). The significant decrease in chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid content was observed after 10 days of treatment with NaCl in *Vicia faba* (Qados and Amira 2011). Similarly, the chlorophyll and total carotenoid contents decreased under salinity treatment in mulberry genotypes (Agastian et al. 2000). At a lower salinity level of 1–2 mS cm^{-1}, the rate of photosynthesis was higher, but it decreased considerably at high salinity levels (Agastian et al. 2000). The growth of *Brassica juncea* was affected under salt stress because of the decreased efficiency of photosystem II (PSII), electron transport rate, and D1 protein (Mittal et al. 2012).

Future studies focusing on understanding the molecular link between salinity and photosynthesis may provide means to identify candidate genes involved in photosynthetic pathways to develop plants with improved salt tolerance.

2.2.3 Water Relation

The direction and the rate of water movement in a plant are regulated by water potential and hydraulic conductivity (Negrao et al. 2017). Salinity in the soil reduces the water uptake by plants due to reduced water potential in the root zone resulting into drought-like conditions (Behboudian et al. 1986; Romero-Aranda et al. 2001). High salinity levels lead to reduction in relative water content in most crops including maize, sorghum, and cucumber (Nagy et al. 1995; Stępień and Kłbus 2006). The leaf water potential, osmotic potential, leaf pressure potential, and relative water content are significantly decreased with increase in salt stress, subsequently affecting many plant processes such as stomatal conductance,

transpiration, net photosynthetic rate, and relative water content (Netondo et al. 2004; Munns and Tester 2008; Sinclair et al. 2013). As the high salt concentration in soil reduces water uptake, it also decreases nutrient uptake by plants, leading to lower NO_3^-, K^+, and Mg^{2+} tissue concentrations (Musyimi et al. 2007; Munns and Tester 2008; Machado and Serralheiro 2017; Sandhu et al. 2017). The effect was more in the mature leaves (Musyimi et al. 2007). An increase in water use efficiency (WUE) upon salt stress is commonly observed, as the reduction in water use (due to reduced stomatal conductance) is more than the reduction in carbon gain (Chartzoulakis and Klapaki 2000).

It is an immense challenge for a plant to maintain ion homeostasis under salinity stress, as accumulations of Na^+or Cl^- interfere with plant's ability to store other ions. During salinity stress, as more ions accumulate in the vacuole, plant tries to balance the decreased water potential in vacuole by accumulating compatible solutes such as proline, glutathione, glycine betaine, and polyols in the cytoplasm (Negrao et al. 2017). Exogenous application of glycine betaine is known to enhance salt tolerance by reducing tissue accumulation of Na^+ (Mäkelä et al. 1998). Accumulation of glycine betaine results in increased Na^+ efflux and decreased K^+ efflux from the plant roots (Wei et al. 2017). Glutathione and proline that play role in maintaining cell turgor and redox homeostasis can act conjointly to allow plants to withstand the joint attack of metalloids and salinity (Anjum et al. 2014). Additionally, accumulation of total soluble sugars increases with increased salinity and plays an important role in osmotic adjustment in plants (Moles et al. 2016).

Some genes that play important roles in plant water relations during salinity have been identified. For instance, the stress-induced aquaporin 1 (AQP1) plays a significant role in improving WUE, hydraulic conductance, and yield under salt stress (Sade et al. 2010; Vysotskaya et al. 2010). The overexpression of AQP1 in tobacco increases leaf net photosynthesis, mesophyll CO_2 conductance, and stomatal conductance, whereas its silencing reduces root hydraulic conductivity under salt stress (Sade et al. 2010). Similarly, reduced hydraulic conductivity in salt-tolerant barley plants as compared to the salt-sensitive plants confirmed the role of aquaporin in salt sensitivity (Vysotskaya et al. 2010). The expression of *Arabidopsis HARDY* (*HRD*) gene, an AP2/ERF-like transcription factor, improved the water use efficiency and ratio of biomass to water use in rice (Karaba et al. 2007).

Identification and manipulation of additional genes involved in water/nutrient uptake and effective compartmentalization may provide new means to improve plants to withstand high salinity levels.

2.3 Mechanism of Salt Tolerance

Salt tolerance mechanism is a complex of various different pathways that work in coordination and are interdependent on each other. Plants develop several biochemical and physiological mechanisms to survive in high-salinity environment. Some of the important aspects of salt stress and important mechanisms responsible for salt tolerance are discussed below.

2.3.1 Tissue Na^+ Accumulation as an Indicator of Salt Tolerance

Na^+ plays an important role during salinity stress in several plant species. High salt concentration in soil normally leads to increased Na^+ uptake by plants, causing tissue toxicity. Besides, as several ion transporters transport both Na^+ and K^+ in roots, increased Na^+ concentration in root zone leads to reduced K^+ uptake. In addition, high concentration of Na^+ also leads to K^+ efflux from the root, resulting in lower K^+ concentration, which may be detrimental for the plant. Disruption of K^+ homeostasis leads to reduced photosynthesis, decreased transpiration, and production of reactive oxygen species; all of which result in reduced yield and productivity. Hence, some studies consider K^+:Na^+ as an important parameter for screening lines for salt tolerance (Chen et al. 2007). However, K^+:Na^+ ratio is not always a true indicator of salt tolerance (Genc et al. 2007), perhaps due to the importance of multiple component traits in the salt tolerance mechanism.

In most plant species, the high concentration of Na^+ is toxic to the cell. However, some plant species are able to cope very well with the moderate concentrations of Na^+ or may even benefit from it (Gattward et al. 2012). In particular, when K^+ is deficient in the medium, beneficial effects of Na^+ are pronounced, suggesting that Na^+ does not strictly substitute K^+(Subbarao et al. 2003). Some members of family Amaranthaceae, such as sugar beet and spinach are shown to utilize Na^+ for growth and development (Kronzucker et al. 2013). These species do not need K^+-deficient conditions to see the benefits provided by Na^+, suggesting that Na^+ may play some additive roles besides replacing K^+ (Ors and Suarez 2016). Physicochemical properties of Na^+ are more or less similar to K^+, making it a good replacement for K^+ in maintaining cell osmoticum (Kronzucker et al. 2013). In addition, the cell K^+ also plays important roles in protein synthesis, oxidative phosphorylation, and as an essential ion for many enzyme functions. Na^+, on the other hand, is known to inhibit protein synthesis and oxidative phosphorylation (Greenway and Osmond 1972). Nevertheless, Na^+ can partially assume some of the roles played by K^+ in the activation of enzymes (Kronzucker et al. 2013). Na^+ is known to improve root nitrate uptake and leaf nitrate assimilation, which may contribute toward reported Na^+ benefits in certain plant species (Kronzucker et al. 2013). It has also been established that Na^+-dependent pyruvate transport into chloroplasts is critical for C_4 habit in some C_4 species, which also explains why Na^+ is an important nutrient for some C_4 species (Weber and von Caemmerer 2010).

Many salt tolerance studies conducted in various crop species focused on studying relationship between tissue Na^+ concentration and salt tolerance (McKimmie and Dobrenz 1991; Genc et al. 2007). However, scientific evidence is mounting to show that the tissue Na^+ concentration may not always represent true picture in terms of salt tolerance (Munns and Tester 2008; Sandhu et al. 2017). Recently, in a salinity study in alfalfa, two genotypes that stored least Na^+ in their leaf tissues in salt treatment were among the worst performers in terms of salt tolerance index among 12 genotypes (Sandhu et al. 2017). On the other hand, two best performers were also low Na^+ accumulators under salt stress. These observations indicate that

although the tissue Na^+ concentration is important, other component traits of the salt tolerance mechanism also play substantial roles (Sandhu et al. 2017). In strawberries and avocadoes, the tissue Cl^- toxicity played a primary role during salinity stress, and Na^+ concentration in leaves does not relate well with the yield performance (Khayyat et al. 2007; Suarez and Grieve 2013; Mauk et al. 2017).

2.3.2 *Na^+ Exclusion from Roots*

The mechanism of Na^+ exclusion from roots is considered pivotal in several crop species (Munns and Tester 2008; Liu et al. 2015). The salt overly sensitive (SOS) pathway that is well characterized in *Arabidopsis* is the key for Na^+ exclusion (Qiu et al. 2002). During salt stress, Ca^{2+} signal is sent by the plant that activates the SOS3 protein (Shi et al. 2002; Gupta and Huang 2014). Active SOS3 binds to SOS2 and stimulates its kinase activity, which in turn phosphorylates SOS1 (Shi et al. 2002). SOS1 is a membrane-associated protein that regulates Na^+ efflux from the root. The manipulation of the SOS proteins have been shown to be successful in enhancing salt tolerance in various plant species (Zhang and Blumwald 2001; Shi et al. 2003; Munns and Tester 2008; Gupta and Huang 2014; Liu et al. 2015).

2.3.3 *Sequestration of Na^+ into Vacuoles*

Even in the presence of an efficient Na^+ exclusion mechanism, high salt concentrations in the soil or irrigation water result in increased Na^+ concentrations in the cytosol. High Na^+ in the cytosol is detrimental to cellular functions. To protect the cytosol from the toxic effects of Na^+, some plants move excessive Na^+ into vacuoles using the Na^+/H^+ exchanger (NHX) proteins. Of the NHX proteins, NHX1 through NHX4 are present in tonoplast and direct Na^+ into vacuole (Barragan et al. 2012). NHX5 and NHX6 target Na^+ into endosomal membranes including vesicles, Golgi, trans-Golgi network, and prevacuolar compartment (Bassil and Blumwald 2014). Several studies targeting the NHX proteins validated important roles of NHX proteins in enhancing salt tolerance in several plant species (Apse et al. 1999; Zhang and Blumwald 2001; Xue et al. 2004; Rajagopal et al. 2007; Bassil et al. 2011; Mishra et al. 2014; Sandhu et al. 2018).

2.3.4 *Enhanced Tissue Tolerance to High Na^+ Concentrations*

In response to high Na^+ concentration, plants produce organic solutes to maintain ion homeostasis. Some of the common solutes that are studied in response to salinity include proline, glycine betaine, and total soluble sugars. Proline accumulation was

far greater in a salt-tolerant genotype of tomato as compared to susceptible one under salt stress (Gharsallah et al. 2016). Proline not only works as an osmolyte, it is an antioxidant and an important component of cell wall (Verbruggen and Hermans 2008; Gharsallah et al. 2016). In addition, proline accumulation in leaves maintains chlorophyll levels and turgor pressure that are critical for photosynthetic activity (Gharsallah et al. 2016; Sandhu et al. 2017). Glycine betaine also plays an important role in ion homeostasis during salt stress (Wei et al. 2017). Treatments involving exogenous application of glycine betaine resulted in enhanced tolerance to salt (Mäkelä et al. 1998), possibly due to its role in reducing K^+ efflux and increasing Na^+ efflux (Wei et al. 2017). It was speculated that increase Na^+ efflux may be regulated through the SOS pathway.

2.3.5 Cl^- Toxicity During Salt Stress

Most salinity studies in plants focus on role of Na^+ in tissue toxicity; however, the role of Cl^- is often disregarded. Now it is well established that for some sensitive plant species, such as avocado, grapes, and strawberries, Cl^- concentration in leaves is better correlated with tissue toxicity (Tregeagle et al. 2010; Suarez and Grieve 2013; Li et al. 2017). Many of the effects of chloride toxicity are hard to differentiate from Na^+ toxicity; however, some effects are unique to Cl^- toxicity (Li et al. 2017).

A number of proteins involved in regulating Cl^- concentration in plants have been characterized in the recent years that include nitrate transporter 1/peptide transporter family proteins (NPFs), homologs of slow anion channel-associated (SLAHs), cation/Cl^- cotransporters (CCCs), cation/H^+ exchangers (CHXs) proteins, aluminum-activated malate transporters (ALMTs), and Cl^- channels (CLCs) (Li et al. 2017). The common mechanism plants use to manage Cl^- concentration in shoot tissue is by abscisic acid (ABA)-mediated Cl^- uploading in the root xylem (Gilliham and Tester 2005). A nitrate transporter 1/peptide transporter family gene, *NPF2.4*, was shown to be involved in catalyzing Cl^- efflux out of cells and regulated xylem uploading (Li et al. 2016a). Knockdown of *AtNPF2.4* resulted in reduction in shoot accumulation of Cl^-, and overexpression of *AtNPF2.4* led to a 23% increase in shoot Cl^-, suggesting an important role of *AtNPF2.4* in regulation of shoot Cl^- concentration during salinity stress (Li et al. 2016a). Similarly, AtSLAH1, a homolog of the slow-type anion channel AtSLAC1, interacts with AtSLAH3 in controlling Cl^- uploading in xylem and regulates root-to-shoot Cl^- transport (Qiu et al. 2016). AtCCC has been shown to be involved in coordinated symport of K^+, Na^+, and Cl^- and played a critical role in plant development (Colmenero-Flores et al. 2007). Under high-salinity environment, the *ccc* mutant plants stored higher Cl^- amounts in shoots and lower Cl^- amounts in roots as compared to the wild-type plants, suggesting its important role in Cl^- homeostasis (Colmenero-Flores et al. 2007).

Some transporters also play role in exclusion of Cl^- from the roots. For instance, NPF2.5 that displayed 83% homology at amino acid level to NPF2.4 was shown to

regulate Cl^- efflux from roots, keeping lower concentration of Cl^- in roots, which in turn resulted in lower Cl^- concentration in shoot (Li et al. 2016b).

Sequestration of Cl^- into the vacuole is an efficient mechanism to keep Cl^- concentration low in the cytoplasm. In *Arabidopsis*, two ALMTs, AtALMT9 and AtALT12, are believed to be involved in Cl^- homeostasis by moving Cl^- across vacuolar membrane (Li et al. 2017). AtALMT9 was originally characterized to be involved in the opening and closing of stomata by regulating Cl^- concentration in the vacuole of guard cells (Baetz et al. 2016). AtALMT9 is now believed to regulate Cl^- concentration in roots cells by moving excess Cl^- to the root vacuole (Baetz et al. 2016). A homolog of AtALMT9, AtALT12, is involved in stomatal closure and is a strong candidate that may have a role in long-distance transport of Cl^- (Sasaki et al. 2010). Similarly, CLCs regulate Cl^- homeostasis by sequestering Cl^- into root and leaf vacuoles. Of the two CLCs known to transport Cl^-, AtCLCc directs Cl^- into root vacuoles, and AtCLCg helps in compartmentalization of Cl^- into vacuoles of mesophyll cells (Jossier et al. 2010; Nguyen et al. 2016). Some CHX proteins can regulate both Na^+ and Cl^- concentrations in plants. For instance, GmSALT3/CHX1 that controls Na^+ and Cl^- accumulation in soybean provides yield advantage by maintaining higher seed weight under saline conditions (Liu et al. 2016).

Additional studies focusing on understanding the role of Cl^- transporters in salt tolerance, specifically in crop plants where Cl^- toxicity is the main cause of salt stress, will help in refining tools and techniques to develop genetic material tolerant to salt.

2.4 Conclusions and Future Perspectives

Traditional breeding approaches had limited success in identifying material tolerant to salinity due to the complexity of the salt tolerance mechanism. Besides, selecting plants under salinity based on biomass resulted in selection for plant vigor. Combining vigor-based selections with selections based on component traits of the salt tolerance mechanism such as ion exclusion, sequestering ions into vacuole, and high tolerance to ion toxicity may improve the potential to obtain genotypes that maintain economically feasible yields and have high salt tolerance. In the last decade, significant progress has been made in understanding plant responses to salinity, and the roles of several important players involved have been elucidated. However, still large gaps exist in the comprehensive understanding of the molecular and biochemical responses in crop plants. Future studies should focus on understanding how intra- and intercellular interactions play role during salinity stress response and how they relate to salinity tolerance at whole plant level. Identification and characterization of novel candidate genes and their utilization may help in developing new salt-tolerant genotypes that are vigorous and high yielding.

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Chapter 3
Marker-Assisted Breeding for Disease Resistance in Crop Plants

Paul Joseph Collins, Zixiang Wen, and Shichen Zhang

Abstract Breeding disease-resistant crop varieties is a cornerstone of disease management. Marker-assisted selection (MAS) incorporates a plethora of plant genomic resources into the process of breeding disease-resistant crops. Although there are species-specific and disease-specific considerations, much of the procedures and theory behind MAS are conserved. Using molecular markers is most likely to increase the efficiency of the breeding process in cases where disease resistance is controlled by one or few genes, and those genes have a large effect on the resistance phenotype. In cases where disease resistance is controlled by many genes of small effect, genomic selection (GS) may be more efficient than MAS or phenotypic selection. GS is an emerging technology, and many of the statistical principles and procedures are still being developed. This chapter should begin to inform breeders as to the potential and the details to consider if using a marker-assisted breeding tool in their plant breeding program.

Keywords Marker-assisted breeding · MAS · Genomic selection · Disease resistance · Soybean cyst nematode · Soybean

3.1 Introduction

The genetic improvement of plant species for the benefit of humanity is an ongoing endeavor with a rich history and hope-filled future. Domestication traits were selected from natural variation within plant populations beginning in prehistory. Farmers continued selecting plant traits which improved agricultural outputs and increased quality for millennia. Beginning in the late nineteenth century, plant genetic improvement began to be practiced in a more organized fashion, now called

P. J. Collins (✉) · Z. Wen
Plant, Soil and Microbial Science, Michigan State University, East Lansing, MI, USA
e-mail: colli490@msu.edu

S. Zhang
Agronomy Department, Kansas State University, Manhattan, KS, USA

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plant breeding programs. These breeding programs were based on simple concepts, induce novel variation through sexual recombination, and select the offspring with the most desirable set of traits. This started as visual selection, but with the advancement of the sciences, it became possible to use scientific information to inform the selection of new plant varieties.

Deoxyribonucleic acid (DNA) was determined to be the molecular basis of heredity in experiments during the twentieth century. DNA is a double helical molecule arranged in strings of sugar phosphate backbones and four possible nucleotides. It is the sequence of these nucleotides which forms the molecular basis of heredity. Every living cell has DNA, which is transcribed into ribonucleic acid (RNA) and translated into a sequence of amino acids, which form proteins. These proteins direct cellular function, causing differences in cellular specialization and leading to the diversity of growth and development observed in all forms of life. Thus, differences in nucleotide sequence account for the stunning array of life variation we observe from humans to insects, to bacteria, and to plants. Variation within a species is also attributed to the differences in DNA sequence.

The goal of a plant breeder is to collect variation within their crop species, identify genetically controlled traits which make some individuals superior, facilitate sexual recombination to reshuffle combinations of DNA sequences, and select the individuals best suited for agricultural production. The underlying knowledge of DNA as the basis of heredity allows plant breeders to take a new approach to this system. First, the DNA sequences which are responsible for important agronomic traits (e.g., disease resistance) are identified. Ideally, the causal gene responsible for the trait is identified. However, the exact gene responsible for the trait may not be identified. Rather, a larger region of the genome is known to be responsible for the trait. This region is commonly called quantitative trait loci (QTL). Next, tools called molecular markers are developed which allowed the breeder to determine if a breeding line contains the desirable DNA sequence. Lastly, the breeder uses the molecular marker data to predict which lines will have superior field performance and uses this prediction in his/her line selection. This multistep system is called marker-assisted selection (MAS).

3.2 Benefits of MAS

MAS has many benefits over phenotypic selection. In many cases, MAS is more cost-efficient than expensive field or greenhouse trials. Also, MAS can be more reliable than phenotypic selection. Phenotypic selection for disease or insect resistance is dependent upon the presence of the disease or insect. However, the environment of the field trial must also be conductive to disease to see symptoms. MAS relies on genetic markers which can be assessed independently on the environment. This means that breeders can use MAS to select for traits outside of the target environment, such as in a winter nursery or in a greenhouse. Breeders are often interested in selecting a new variety with multiple independent resistance genes. This is referred to as “gene pyramiding” or “gene stacking” and is likely to delay or prevent

Line Name	Line 1	Line 2	Line 3	Line 4
Genotype	No Res Genes	One Res Gene	Two Res Genes	Three Res Genes
Phenotype	Susceptible	Resistant	Resistant	Resistant
Visual				

Fig. 3.1 Demonstration of how MAS can be beneficial when attempting to stack multiple genes which result in the same phenotype. In the table, phenotypic selection would equally value Line 2, Line 3, and Line 4. However, MAS would inform the breeder that Line 4 has a stack of three resistance genes, and would be the best selection

the pathogen from overcoming the host resistance. With phenotypic selection, gene pyramiding is difficult because the presence of one resistance gene confounds the detection of additional resistance genes (Fig. 3.1). MAS allows the breeders to select for multiple independent resistance genes and stack them into a variety with more resilient resistance. Additionally, MAS can allow the breeder to select for some traits earlier in the breeding pipeline. The early culling of breeding lines without the desired resistance genes, ensures that the lines being evaluated in later stage of the breeding pipeline have good potential. Early generation selection ensures that breeding pipeline resources, such as expensive yield testing, are used efficiently.

3.3 Limits of MAS

Although MAS has many benefits over phenotypic selection, there are also limits to the applicability to MAS. Firstly, the causal gene(s) (or a narrowly defined QTL) for the trait must be known. This can be determined by genetic mapping or identified from the scientific literature. Given that the genetic resources are available, molecular markers can be designed to be closely linked to the causal gene(s) or QTL. If the molecular marker is not close to the causal gene, there is a larger possibility of a meiotic crossover event occurring between the marker and the gene. In this case, MAS will fail to identify the breeding lines with the causal gene, and the molecular marker will be said to be "broken." One way to decrease the likelihood of recombination events breaking the marker is to use multiple molecular markers which flank the causal gene. There is still a possibility that a double crossover could break both flanking markers from the causal gene, but this is rare.

Additionally, for MAS to be effective, the causal gene(s) need to account for a large effect of the phenotypic variance. This is not always the case, and using MAS on a gene which accounts for a small percentage of the phenotypic variance may result in limited selection gains. The effect of causal genes can also be confounded by genetic x environment interaction. Sometimes, causal genes may perform differently in different genetic backgrounds. For these reasons, caution should be taken when implementing a MAS in a breeding program, and the breeder should periodically confirm that the selections carry the desired trait.

3.4 Case Study of MAS

The following section will use a case study of a breeding program using MAS to illustrate the steps and general procedures of MAS. The case study focuses on marker development, parental and progeny screening, and line selection in the Soybean Breeding Program at Michigan State University for the trait of soybean cyst nematode (SCN) resistance. Although many of the details are unique to this crop and disease, the principles illustrated are easily translated to many crops and disease resistance traits.

3.4.1 SCN Resistance Breeding

Soybean cyst nematode (SCN) (*Heterodera glycines*) has been considered the most important pest in soybean production worldwide. SCN causes yield losses by feeding on roots, leading to nutrition loss, retarding root growth, inhibiting *Bradyrhizobium japonicum* nodulation (Riggs and Schmidtt 1987), and creating wounds for other pathogen invasion (e.g., *Fusarium virguliforme*) (Roy 1997).

Breeders have made significant efforts in developing resistant varieties; the resistance in most of the released varieties is traced back to "Forrest," "Peking," and PI 88788. To date, SoyBase (Grant et al. 2010) has included 207 QTLs identified by numerous QTL mapping studies. Some of these QTLs likely are the same QTLs with different alleles as they share similar genomic regions (Concibido et al. 2004). Among all the mapped QTLs, Rhg1 and Rhg4 represent the major sources of SCN resistance, and the causal genes have been functionally characterized (Cook et al. 2012; Liu et al. 2012).

Traditionally, breeders phenotypically select resistant lines by using a greenhouse-based SCN bioassay, which is costly and labor intensive. Also, it is tedious when selecting for lines with a stack of multiple resistance genes against difference races of SCN. Currently, most soybean breeders have switched to using MAS as a cost-effective tool to breed for SCN-resistant soybeans. Studies have shown MAS using flanking markers has 98% accuracy in identifying resistance lines (Mudge et al. 1997).

The Soybean Breeding and Genetics Lab at Michigan State University has converted single nucleotide polymorphisms (SNP) closely linked to the Rhg1 and Rhg4 genes (Cook et al. 2012; Liu et al. 2012; Shi et al. 2015) to KASP™ markers, including "Rhg1-1," "Rhg1-2," "Rhg1-3," "Rhg4-5," and "ShiRhg1-1," to routinely assist selections for resistance against different SCN races. The sequence information is listed in Table 3.1.

In 2015, seven breeding populations were screened for SCN resistance and aphid resistance (Table 3.2). Genomic DNA of parental lines were extracted with the modified CTAB protocol (Kisha et al. 1997) with using 15 ml tubes. Two replications of parental test were conducted to screen for polymorphic markers (Fig. 3.2). Marker

Table 3.1 KASP™ markers designed for screening for SCN resistance

Marker ID	Primer allele FAM	Primer allele VIC	Primer common	Allele FAM	Allele VIC
Rhg1-1	GCAGGACACAACAAAGTATAATCAGG	AGCAGGACACAACAAAGTATAAT CAGA	CATAGTAGCAGGAGTTCTCC TCGTA	C	T
Rhg1-2	CCACCTGCTTCAACATCACCAC	CCACCTGCTTCAACATCACCAG	GACAGAAAAAGGAGTCCCTCC AGTT	G	C
Rhg1-3	GAGGCAAATCTAGTCCATTCATAGA	GAGGCAAATCTAGTCCATTCATAGG	GTTATCTTGTTAGAACTCCTAACG AGGAA	T	C
Rhg4-5	TAAAACCCTCAAACATACTACTAGGT	AAAACCCTCAAACATACTACTAGGC	AAATCAGCAACCTTTGCTCTCAT GTCAT	T	C
ShiRhg1-1	GAAGGTGACCAAGTTCATGCTAGCCA AAGAACTTGAGSAGATGAG	GAAGGTCGGAGTCAACGGATTAGCCA AAGAACTTGAGSAGATGAT	CAAACAATAGGTCCAACCACCA	G	C

Table 3.2 Integrating SCN resistance and aphid resistance in soybean advanced breeding lines with using MAS. *More information about flanking markers of aphid resistance genes, Rag6 and Rag3c, can be found in Zhang et al. (2017a, b)

Cross ID	Female	Male	Breeding goals	Traits carried by female	Traits carried by male
130094	14P051	14P004	Rag3c, Rag6, SCN	Rag3c, Rag6	SCN
130095	14P052	14P004	Rag3c, Rag6, SCN	Rag3c, Rag6	SCN
130105	14P055	14P004	Rag3c, Rag6, SCN	Rag3c, Rag6	SCN
130133	14P004	14P059	Rag6, Rag3c, SCN	SCN	Rag6, Rag3c
130134	14P004	14P060	SCN, Rag6, Rag3c	SCN	Rag6, Rag3c
130144	14P002	14P059	Peking SCN R to HG 2.5.7, Rag6, Rag3c	SCN	Rag6, Rag3c
130169	14P058	14P004	Rag6, Rag3c, SCN	Rag6, Rag3c	SCN
130170	14P059	14P004	Rag6, Rag3c, SCN	Rag6, Rag3c	SCN

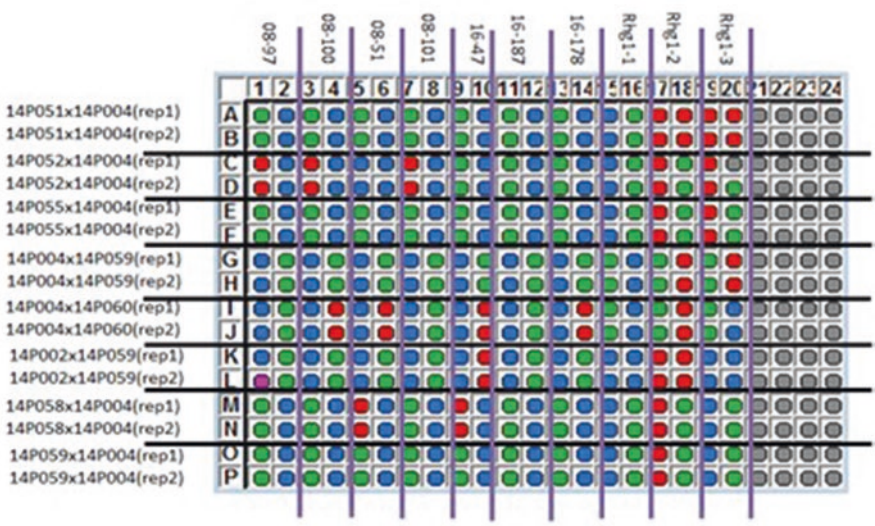

Fig. 3.2 Parental test to screen for polymorphic markers for each breeding populations. Green represents the y-allele. Blue represents the x-allele. Red represents the heterozygote (both alleles). Pink represents unknown allele, which is due to imperfect PCR reaction. Gray represents negative PCR reaction

"Rhg1-1" is polymorphic in all breeding populations except the cross 14P002 X 14P059. As for marker "Rhg1-2," at least one parent of each population showed heterozygous status, which could be due to an imperfect marker design or the heterozygote nature of the loci in the parent lines. Marker "Rhg1-2" was polymorphic in four breeding populations; however, in the other four populations, one or both parents were heterozygous at the SNP locus.

DNA of each plant from the F_3 breeding populations was extracted with a high-throughput CTAB protocol (Zhang et al. 2017b) and screened with polymorphic

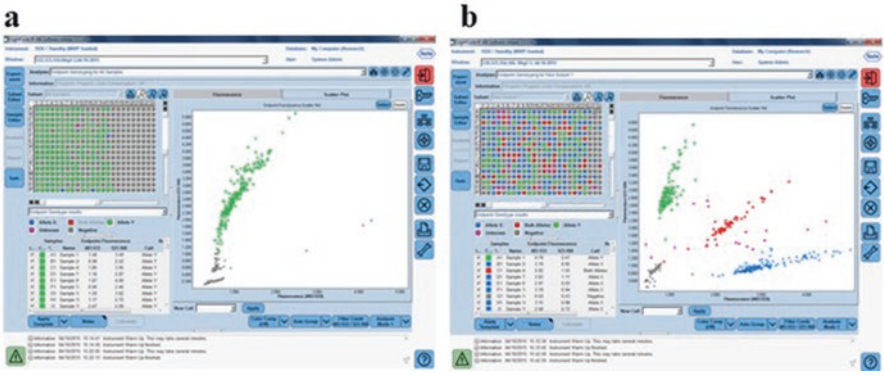

Fig. 3.3 Examples of a segregating population and a nonsegregating population tested with polymorphic markers. Rhg1-1 segregated perfectly in population A. Rhg1-3 had no segregation in population B because the population was likely derived from a false-positive cross

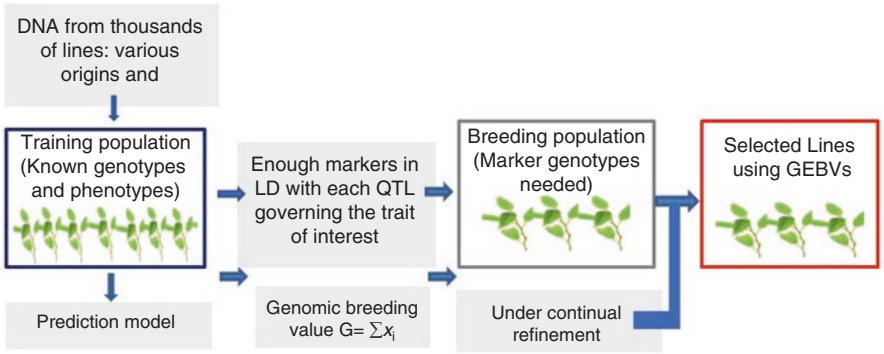

Fig. 3.4 Flowchart showing the basic steps of genomic selection (GS). Collection of both phenotypic and genotypic data for each line in the training population (TP). Integrating the genotypic and phenotypic data, conduct statistical analysis on TP to estimate allele effects at all loci on the phenotype. Generation of a prediction model for the GEBVs that combines all the marker genotypes with their effects on the predictive value of each line. Application of the prediction model on breeding population for which genotypes (but not phenotypes) are available. GEBVs are estimated and the best lines are selected for breeding

KASP™ markers (Zhang et al. 2017a). Most polymorphic markers segregated perfectly in the progenies (Fig. 3.3a). However, few of them had no segregation (Fig. 3.3b), which was due to the false-positive crosses. Soybean is a self-pollinating crop that completes selfing before flowers open. Breeders usually make crosses on closed flowing buds. Therefore, false-positive crosses occur when the bud selfed before pollinating by other pollen. When compared to the traditional bioassay trails, MAS can be cost-effective and efficient in detecting these types of false crosses (Fig. 3.4).

For each marker, the favorable allele was determined based on the genotype of the favorable parent. As shown in Table 3.3, progenies with favorable alleles were

Table 3.3 Using marker data to inform selections for SCN and aphid resistance in a small subset of an F_3 breeding population

Breeding goals	SCN resistance		Aphid resistance		
Markers	Rhg1-1	Rhg1-3	08-100	08-101	16-178
Favorable allele	Allele T	Allele T	Allele T	Allele T	Allele T
1	Allele X	Allele X	Allele T	Allele T	Allele T
2	Allele X	Allele X	Allele T	Allele T	Allele T
3	Allele T	Allele T	Both Alleles	Both Alleles	Allele X
4	Allele X	Allele X	Both Alleles	Both Alleles	Both Alleles
5	Allele X	Allele X	Both Alleles	Both Alleles	Both Alleles
6	Both Alleles	Both Alleles	Both Alleles	Both Alleles	Allele T
7	Both Alleles	Both Alleles	Allele T	Allele T	Allele X
8	Allele X	Allele X	Allele X	Allele X	Allele T
9	Allele X	Allele X	Both Alleles	Both Alleles	Both Alleles
10	Both Alleles	Both Alleles	Negative	Both Alleles	Allele T
11	Allele X	Negative	Both Alleles	Both Alleles	Allele T
12	Allele T	Allele T	Allele X	Allele X	Allele T
13	Allele T	Allele T	Both Alleles	Both Alleles	Allele T
14	Allele T	Allele T	Allele T	Allele T	Both Alleles
15	Allele T	Allele T	Allele X	Allele X	Both Alleles
16	Allele T	Allele T	Allele T	Allele T	Unknown
17	Allele T	Allele T	Allele X	Allele X	Allele T
18	Allele T	Allele T	Allele X	Allele X	Both Alleles
19	Allele X	Allele X	Allele T	Allele T	Allele T
20	Allele X	Allele X	Both Alleles	Both Alleles	Allele T
21	Allele T	Allele T	Allele X	Allele X	Allele X
22	Allele X	Negative	Allele T	Negative	Allele T
23	Allele T	Allele T	Allele T	Allele T	Allele T
24	Allele X	Allele X	Allele X	Allele X	Negative
25	Allele T	Allele T	Both Alleles	Both Alleles	Allele X

selected. Line 23 had favorable alleles at all targeted SNPs, suggesting it is a promising breeding line with resistance to both SCN and aphids. All selected lines were later tested with SCN and/or aphids and showed strong resistance, indicating the effectiveness of MAS.

3.5 Marker Discovery

In the past decade, massively parallel sequencing platforms have become wildly available, which have made producing molecular markers cost-effective. Whole-genome resequencing, RNA sequencing, whole-genome exome capture sequencing, and reduced representation sequencing (e.g., restriction site-associated DNA sequencing, genotyping by sequencing, specific-locus amplified fragment sequencing) with or without a reference genome are all advances in recent years which facilitate the discovery of SNPs and presence/absence variation (PAV) (Baird et al. 2008; Huang et al. 2009; Wang et al. 2009; Davey et al. 2011; DePristo et al. 2011; Peterson et al. 2012; Poland and Rife 2012). Once SNPs and/or PAVs are identified, markers can be designed to detect the variation.

In 2010, a high-quality soybean (*Glycine max*) reference genome was built by sequencing the variety "Williams 82" with whole-genome shotgun sequencing approach (Schmutz et al. 2010). With the availability of the soybean reference genome, Glyma.Wm82, SNPs, and PAVs have been efficiently discovered by mapping the sequencing reads from diverse soybean genotypes to the reference genome. Song et al. (2013) sequenced reduced representation libraries from six cultivated and two wild soybean (*G. soja*) genotypes; a total of 52,041 SNPs were identified and used to design the SoySNP50K iSelect BeadChip. A mapping population

consisting of 246 recombinant inbred lines were sequenced at an average of 0.19× depth and 109,273 SNPs were identified and used to map QTLs underlying resistance to southern root-knot nematode (Xu et al. 2013). Li et al. (2014) built the pan-genome of *G. soja* by de novo assembly of seven diverse *G. soja* accessions, and variations (e.g., SNPs) between Glyma.Wm82 reference genome and *G. soja* genomes were identified; this assembly-based method provided more SNPs than by resequencing because reads from divergent regions are difficult to be mapped to the Glyma.Wm82 reference genome. A whole-genome exome capture sequencing approach was used by Zhang et al. (2017b) in detecting sequence variations (including SNPs) on exons of candidate genes for aphid resistance.

Using the data from genetic mapping studies and the SNP resources identified, SNP assays can be developed for use in MAS. A customized genotyping system can be developed using customizable assays from several commercial biotechnology companies. Common assays include the Illumina GoldenGate (Fan et al. 2006), Kompetitive Allele Specific PCR (KASP™) (LGC, Middlesex, UK), and TaqMan® (Life Technologies, Carlsbad, CA, USA).

3.6 Confirmation of Parental Polymorphism

A common issue with using true SNPs that were discovered from a different panel than the application panel is the ascertainment bias (Heslot et al. 2013). In other words, SNPs discovered from a certain panel of genotypes may not be polymorphic in breeding populations that are genetically distinctive. Therefore, parental lines should be tested for marker polymorphism before conducting MAS in a breeding population. In some cases, SNPs that are polymorphic between the parental lines are not segregating perfectly in the breeding population due to some unknown biological process (e.g., gametic selection) or from a false-positive cross (the female parent was self-pollinated before it was pollinated by the male parent's pollen). To avoid unnecessary genotyping cost of theses pseudo-polymorphic markers, a random small sample of progeny (~30 individuals) can be tested for polymorphism before screening a large breeding population.

3.7 High-Throughput Marker Screening

In breeding programs, thousands of lines need to be screened for multiple traits. A high-throughput DNA extraction and genotyping system is needed. At the Soybean Genetics and Breeding Laboratory of Michigan State University, a high-throughput DNA isolation pipeline with using 96-well plates was developed following the modified CTAB protocol (Kisha et al. 1997). Briefly speaking, DNA of breeding lines were isolated in parallel on 96-well plates with extraction volume scaled down to 400 μL (Zhang et al. 2017b). After normalizing the DNA concentration to 20–150 ng/μL, each DNA sample was genotyped with KASP™ or TaqMan® genotyping

assays in parallel on 384-well plates, and genotype calling was performed using the Endpoint Analysis module of LightCycler® 480. Detailed protocols of TaqMan® and KASP™ genotyping were described by Zhang et al. (2014, 2017a).

3.8 Using Marker Data to Inform Selections

Compared to the traditional phenotypic selection, MAS increases efficiency and precision in selecting favorable plants. Usually, two flanking markers of a causal gene are used to inform selections and progenies with recombination events are discarded. If the distance between the marker and the targeted causal gene is small (recombination is less likely to occur), one marker may be sufficient to inform the selection.

Most breeding programs try to integrate as many beneficial traits as possible. When using marker data to inform multiple selections in the progenies, the most important step is having a good record of the favorable allele of every marker, which is the allele of the parent carrying the beneficial trait. Selection schemes are similar to that of the traditional phenotypic selection, including truncation selection, index selection, tandem selection, and more.

MAS is also commonly used in backcrossing programs to help select for favorable alleles from the donor parent (foreground selection) and recover the genome of the recurrent parent (background selection) (Collard and Mackill 2008). Using markers which cover the background genome, MAS can accelerate the recovery of much of the recurrent parent genome in three backcross generations compared to six generations by the traditional phenotypic selection (Frisch et al. 1999).

3.9 MAS Considerations

Although the case study illustrated the general procedures of employing MAS in a breeding program, there are many other crop-specific and pest-specific considerations to take into account when using MAS. Specifically, the reproductive system of the crop, and the nature of the host-pathogen relationship should be considered. The method of propagation of the crop is important. Some crops, such as soybean and maize, as propagated by seed, whereas other crops, such as potato or cherry, are propagated clonally. With seed propagated crops, one must consider that the plants in the farmers' fields are not genetically identical to the breeding lines, as will be true with vegetatively propagated crops. There is also a large distinction made between self-pollinated crops and outcrossing crops. Self-pollinated crops, like soybean and wheat, are planted in the farmer's field as inbred lines. Inbred lines are almost completely homozygous, meaning that the genetics change little from generation to generation. Many outcrossing species, like maize, are planted as hybrids. These lines are highly heterozygous, as they result from the fertilization of two inbred parental lines. If the breeder is breeding an inbred crop, it is important to use

MAS with a complete focus on additive genetic variance, whereas a breeder for a hybrid crop must consider both additive and dominance effects.

Host plant resistance to disease can be characterized as vertical resistance or horizontal resistance. While there are many factors which affect how the disease resistance is classified, the most relevant distinction is the number of genes present which are responsible for the resistance phenotype. Vertical resistance is characterized by a single gene which causes resistance in the host. Therefore, vertical resistance genes account for a large percentage of the phenotypic variance and are good candidates for MAS. In contrast, horizontal resistance is characterized by many genes of small effect which control resistance to the pest. Because horizontal resistance genes account for less of the phenotypic variance, using them in a MAS program would result in less genetic gain. However, horizontal resistance often has the benefit of being more resilient than vertical resistance. Pests tend to overcome vertical resistance genes because vertical resistance genes are often widely employed, leading to a strong pressure on the pest population. Rare pest genotypes which have overcome the vertical resistance genes become predominant. To prevent this, breeders will try to discover and use multiple vertical resistance genes (either as a stacked trait or in different years). These strategies are thought to reduce the pressure on the pest population and prevent resistance genes from being overcome. The other strategy is for breeders to use horizontal resistance genes. This will require larger populations, and although MAS is possible for breeding horizontal resistance, it is often less effective than phenotypic selection.

3.10 Genomic Selection in Crop Breeding

Marker-assisted selection (MAS) has been used in many plant breeding programs in the last two decades. However, its utilization focuses on traits that are controlled by major genes or fewer numbers of QTLs. For complex quantitative traits which are governed by large number of small-effect QTLs, the method may be inferior to conventional phenotypic selection (Zhao et al. 2014). Moreover, gene interactions (epistasis) and genotype × environment interactions (G × E) have limited the transferability of QTL effects estimates across populations and environments (Xu and Crouch 2008). Researchers have developed genomic selection (GS), a new tool to address these issues.

The strategy of GS, proposed by Meuwissen et al. 2001 estimates the genetic merit of an individual based on molecular genetic information by simultaneously accounting for all DNA markers across a whole genome. GS is a form of MAS that selects progeny lines or parents based on the estimated genomic estimated breeding values (GEBVs), which leads to shorter breeding cycle duration as it is no longer necessary to wait for late filial generations' performance trial. GS needs a large number of molecular markers covering the entire genome. The number of markers needed to cover the whole genome is dependent upon the linkage disequilibrium in the population of interest.

3.11 Statistics of Prediction Models

The standard linear model equation can be formulated as:

$$y = \sum_{n}^{j=1} x_{ij}\beta_j + e_i$$

where y is a phenotype value of the ith individual, x_{ij} represents the genotype of the ith individual at the jth marker of 1 to n markers, β_j is the allelic substitution effect at jth marker, and e is the vector of random residual effects of ith individual. In x_i, the allelic state of individuals can be coded as a matrix of 0, 1, or 2 to a diploid genotype value of AA, AB, or BB, respectively. Since the number of markers serving as predictors (p) is usually far greater than the number of individual lines (n), it raises issues in multicollinearity and overfitting among predictors. Basically, there are two regressions methods, parametric and nonparametric regressions, including 11 models have been developed to address the "large p small n" issue. They are RR-BLUP (Meuwissen et al. 2001), least absolute shrinkage and selection operator (LASSO, Li and Sillanpää 2012), elastic net (EN, Zou and Hastie 2005), Bayesian ridge regression (BRR, de los Campos et al. 2013a, b), Bayesian version of LASSO (BL, Li and Sillanpää 2012), Bayes A (de los Campos et al. 2013a, b), Bayes B (Heffner et al. 2011a, b, c), Bayes C (de los Campos et al. 2013a, b), and $\text{Bayes}_{C\pi}$ (Habier et al. 2011), reproducing kernel Hilbert spaces regression (RKHS, Gianola and van Kaam 2008), and random forest (RF, Holliday et al. 2012).

Heslot et al. (2012) compared these models and suggested that GS in plant breeding programs could be based on a reduced set of models such as the Bayesian LASSO, weighted Bayesian shrinkage regression (wBSR, a fast version of Bayes B), and random forest (RF) (a machine learning method that could capture nonadditive effects).

3.12 Factors Affecting Prediction Accuracy

Genomic selection (GS) efforts have been performed for various traits in different crops and trees including rice, wheat, maize, soybean, canola, alfalfa, *Miscanthus*, switchgrass, grapevine, pine, and eucalyptus. The prediction accuracy ranges from 0.21 to 0.90 for different traits among different plants (reviewer by Nakaya and Isobe 2012; Bhat et al. 2016). Previous studies showed that the accuracy of GS depends on various factors in term of a complex and comprehensive manner. These factors include training population size, heritability of the trait, distribution of QTL effects, and the extent of LD between markers and QTL.

3.12.1 Training Population Size

Since marker effects are estimated from the number of individuals with phenotypes and genotypes in the training set, usually the prediction accuracy increases as the training population size increases. For example, an increase in the TP of a soybean landrace population (TP comprising of 97, 147, 197, and 247 lines) and a multifamily soybean breeding program (TP comprising of 0 to 250 lines) resulted in a consistent increase in the prediction accuracy for grain yield (0 to 0.54) (Jarquin et al. 2014; Zhang et al. 2016). Similar findings were also confirmed by two wheat populations (Heffner et al. 2011a, b, c; Ornella et al. 2012).

3.12.2 The Extent of LD Between the Markers and the QTL

Given that complex quantitative traits, such as horizontal disease resistance, are governed by a large number of small-effect QTL, it is necessary to use genome-wide high-density markers that can potentially explain all the genetic variance in GS model. How many markers can be considered as "high density"? Usually, the number of markers required for GS modeling is determined based on the extent of linkage disequilibrium (LD) between the markers and the QTL. In other words, you should make sure that at least one marker is in LD with each QTL governing the trait of interest to conserve marker-QTL associations.

For example, the genome size of soybean is around 1000 Mbp (Schmutz et al. 2010), and the marker interval with a significant LD block was ~200 kb (Wen et al. 2015) in 342 soybean landraces. If this landrace population was used for GS modeling, the number of markers required would be at least 5000 (200 kp per SNP). Note that the extent of LD in soybean is similar to that of the self-pollinated species rice (~123–167 kb) and sorghum (~150 kb) (Huang et al. 2010; Morris et al. 2013) but much greater than in maize (1–10 kb), an outcrossing species (Yan et al. 2009). That means outcrossing species always need more markers to secure optimal prediction accuracy than self-pollinated species.

3.12.3 Trait Heritability

Usually high-heritability traits are positively correlated with higher GEBV prediction. Ornella et al. (2012) found that prediction accuracy for wheat resistance to yellow and stem rust was influenced by their low and high heritability. Similar results were noted for plant height (low heritability) and maturity (high heritability) in soybean; the respective prediction accuracy was estimated to be 0.45 and 0.68 (Jarquin et al. 2014). However, there are some irregularities that have yet to be explained. For instance, prediction accuracy for plant height (heritability =0.80) and

grain yield (heritability = 0.69) was 0.45 and 0.64, respectively, in soybean advanced breeding lines (Jarquin et al. 2014).

Moreover, Marulanda et al. (2015) found that variability in prediction accuracy decreased when the heritability increased. For example, for a heritability of 0.4, the prediction accuracy ranged between 0.39 and 0.80 for TP size of 100, whereas for a high heritability of 0.8, the prediction accuracy varied between 0.75 and 0.90 with same TP size.

3.12.4 Number of QTL and the Corresponding Effects

The number of QTL and the corresponding effects will also influence the prediction accuracy. The predication accuracy seems to be negatively related to the number of QTL (Zhong et al. 2009). Furthermore, using real data from a wheat data set and simulating scenarios with a small number of quantitative trait loci (QTL) (20), a moderate number of QTL (60, 180) and an extreme number of QTL (540), Wang et al. (2015) showed that if a small amount of loci had a large effect on a trait, great differences were found between the predictive ability of various methods and BayesCπ was recommended. If a trait was controlled by a moderate number of genes, the absolute differences between the various methods were small, but BayesA was also found to be the most accurate method. Furthermore, BayesA was widely adaptable and could perform well with different numbers of QTL. If a trait was controlled by an extreme number of minor genes, almost no significant differences were detected between the predictive ability of various methods, but RR-BLUP slightly outperformed the others in both simulated scenarios and real data analysis, thus demonstrating its robustness and indicating that it was quite effective in this case.

3.13 Conclusions

Plant breeding for disease resistance is essential to helping increase the production and sustainability of modern agricultural systems. Molecular markers are tools that have been available to aid this process for a few decades. Plant breeders have incorporated molecular markers into their breeding programs using MAS and GS. MAS has been tested and demonstrated over the past two decades and been demonstrated to be effective at selecting new varieties with resistance to plant diseases. MAS is specifically effective for breeding vertical resistance to diseases. However, MAS is less effective for breeding horizontal resistance. The durability of horizontal resistance makes it an attractive target for breeders. GS is a new tool that is in early stages of development, but has the potential to be implemented in breeding programs to breed for many traits, including horizontal resistance. More technological advances are likely to occur, and future breeders can use all tools available to them to continue the process of breeding new varieties to meet the agricultural demands of the future.

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Chapter 4
Morpho-Physiological Traits and Molecular Intricacies Associated with Tolerance to Combined Drought and Pathogen Stress in Plants

Vadivelmurugan Irulappan and Muthappa Senthil-Kumar

Abstract Crops in field conditions are challenged by the simultaneous occurrence of drought and pathogen stress. In the past, research was primarily focused on studying the impact of individual stresses on plants and selection of crop varieties potentially tolerant to particular stress by yield-associated morpho-physiological traits. However, several molecular responses of crop plants underlying morpho-physiological features to concurrent stresses are not similar to that of individual stresses. Certain morpho-physiological traits such as cell membrane stability, leaf water potential, stomatal movement, and root length were shown to be altered distinctly under combined stress to combat the stress condition. However, the relevance of such traits under combined stress tolerance is not precisely known. In this chapter, from the extensive literature survey, we identified several morpho-physiological changes that could be cognate with better plant performance under combined stress and represented them as traits that have potential to impart combined stress tolerance. We have comprehensively explained physiological and molecular basis for each trait and, where possible, suggested the ways to exploit the information for identification of varieties with prospective traits. Also, we proposed the need for systematically studying the underlying regulatory traits under combined stress conditions in the future.

Keywords Combined stress · Drought · Pathogen infection · Morpho-physiological traits · Combined stress tolerance

V. Irulappan · M. Senthil-Kumar (✉)
National Institute of Plant Genome Research, New Delhi, India
e-mail: vadivelmi@nipgr.ac.in; skmuthappa@nipgr.ac.in

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4.1 Introduction

In field condition, crop plants encounter a combination of drought and pathogen infection, which affects their growth and yield more than the respective individual stress conditions (Atkinson and Urwin 2012; Suzuki et al. 2014; Ramegowda and Senthil-Kumar 2015; Pandey et al. 2015). Plant responses to combined stress are quite complex and different from that of respective individual stress conditions (Atkinson and Urwin 2012; Suzuki et al. 2014; Gupta and Senthil-Kumar 2017; Pandey et al. 2017). Under combined drought and pathogen infection, drought influences pathogen infection by predisposing or enduring the plant to infection (Pandey et al. 2017). For instance, *Fusarium oxysporum* f. sp. *ciceris* (causal agent of fusarium wilt), *Rhizoctonia bataticola* (causal agent of dry root rot), and *F. solani* (causal agent of black root rot) infections occur in *Cicer arietinum* (chickpea) mainly under drought stress. In contrast, *R. solani* (causal agent of wet root rot) infection is less prevalent under drought stress (Cook and Papendick 1972; Blanco-Lopez 1983; Landa et al. 2004; Sharma et al. 2015; Jiménez-Díaz et al. 2015). Further, drought predisposes the plants to foliar pathogens such as *Pseudomonas syringe* (causal agent of bacterial speck) on *Arabidopsis thaliana* (thale cress or *Arabidopsis*) (Gupta et al. 2016). Gupta et al. (2016) demonstrated that *Arabidopsis* plants showed reduced *P. syringe* pathogen multiplication under drought stress compared to pathogen-only infected plants. Similarly, pathogen also influences drought-induced changes in plants under combined drought and pathogen infection (Dossa et al. 2017). For example, *Uromyces phaseoli* (causal agent of rust) infection disrupts cuticular layer and prevents stomatal closure in *Phaseolus vulgaris* (bean), which aggravates water loss under subsequent drought stress (Duniway and Durbin 1971).

Plant response to combined stress cannot be understood directly from individual stress studies. For example, stomatal closure is a plant response to individual drought and foliar pathogen infection to reduce transpirational water loss and stop pathogen entry, respectively (Melotto et al. 2006; Grimmer et al. 2012). However, under combined drought and foliar bacterial infection, plant response, in case of stomatal movement, may or may not be similar. Further, *Zea mays* (maize) subjected to drought stress increased its cuticular wax deposition to prevent water loss (Ristic and Jenks 2002). Also, it was shown that maize with reduced cuticular wax deposition was found susceptible to *Clavibacter michiganensis* subsp. *Nebraskensis* (causal agent of Goss's wilt) infection (Marcell and Beattie 2002). This study implies that when maize is subjected to combined stress, plants with high cuticular wax deposition might provide resistance to pathogen infection. However, regulation of wax deposition, as well as composition under combined drought and pathogen infection, is not known.

Various morpho-physiological traits have been attributed to impart individual drought or pathogen stress tolerance, but these traits are not sufficiently studied under combined stress. In this chapter, based on the preliminary evidence described in the literature, we describe a few exploitable morpho-physiological traits, namely, cell membrane stability, leaf water potential, stomatal movement, and root length,

and explain the importance of these traits under combined drought and pathogen infection. Besides, we describe few traits which are affected by combined stress such as leaf area, leaf greenness, canopy temperature, and anthesis. Additionally, traits described in individual stress studies, which have the potential for consideration under combined stress are also explained.

4.2 Morpho–Physiological Traits that Likely Govern Plant Response to Combined Stress

4.2.1 Cell Membrane Stability

Plants undergo changes in cell membrane composition and structure under drought and pathogen infection (Tripathy et al. 2000; Bajji et al. 2002; Pétriacq et al. 2016). Measurement of electrolyte leakage is routinely employed as a useful tool to assess cell membrane stability of plant cells under drought and pathogen infection (Tripathy et al. 2000; Govrin and Levine 2000; Bajji et al. 2002). *Triticum aestivum* (wheat) subjected to drought stress had more electrolyte leakage compared to well-watered plants, and it was ascribed to cell membrane damage due to drought stress (Tripathy et al. 2000; Bajji et al. 2002). *Arabidopsis* plants infected with *Plectosphaerella cucumerina* (causal agent of blight) had more electrolyte leakage as compared to mock-inoculated plants. Also, electrolyte leakage was increased with inoculum size as cell death was positively correlated with the inoculum concentration (Pétriacq et al. 2016). These individual stress studies indicate that plants with high cell membrane integrity can be tolerant to individual drought or pathogen infection. Similarly, the role of cell membrane stability in combined stress has been hinted (Gupta et al. 2016; Ramu et al. 2016). Under combined drought stress and *P. syringae* infection, *Arabidopsis* plants showed increased electrolyte leakage as compared to the plants subjected to individual stress conditions. Additionally, *Arabidopsis* plants subjected to drought stress followed by *P. syringae* infection and vice versa had high and low electrolyte leakage, respectively. High electrolyte leakage was associated with reduced *in planta* bacterial number in plants subjected to combined stress as compared to only infected plants, (Gupta et al. 2016). These studies show that plant response varies with stress chronology under combined stress. Ramu et al. (2016) showed that *Helianthus annuus* (sunflower) subjected to combined drought and *Plasmopara halstedii* (causal agent of downy mildew) infection had increased malondialdehyde content, an indication of cell membrane damage, as compared to plants exposed to individual stresses (Ramu et al. 2016). These studies indicate that plant response vary with plant and pathogen type. Quantitative trait loci (QTL) governing cell membrane stability in wheat under drought stress is known (Tripathy et al. 2000; Loarce et al. 2016) (Table 4.1). However, QTL variation under combined stress condition is yet to be identified.

Table 4.1 Quantitative trait loci (QTL) linked with exploitable morpho-physiological traits under individual drought stress[b]

Sl. no	Trait	Plant name	QTL[a]	QTL flanking marker	Reference
1	Cell membrane stability	*Oryza sativa*	QCMS3.1	EM11_2 – RZ403	Tripathy et al. (2000)
2	Leaf water potential	*O. sativa*	qDTY 12.1	RM28048- RM511	Bernier et al. (2009) Shamsudin et al. (2016)
3	Stomatal frequency	*Triticum aestivum*	Qsl-5A.1	Xwmc410–Xwmc74	Wang et al. (2016)
4	Root length	*T. aestivum*	Qrls. Uwa.3AL	Xgwm391-Xbcd1431	Ayalew et al. (2017)

[a]Only QTLs with high phenotypic variation are listed
[b]Representative list with select examples that are suitable for combined stress only

4.2.2 Leaf Water Potential

Under drought and pathogen infection, plants undergo changes in leaf water potential, which is a result of an increase in hydraulic resistance and cell turgor loss (Paul and Ayres 1984; Yan et al. 2017). Leaf water potential was exploited as a direct physiological parameter to estimate plant water status under combined stress (McElrone et al. 2003). Effect of individual drought and *Xylella fastidiosa* (causal agent of bacterial leaf scorch) infection and combined stress on leaf water potential of *Parthenocissus quinquefolia* (Virginia creeper) was demonstrated. Plants subjected to combined stress had low water potential as compared to plants exposed to individual stresses. The low water potential was attributed to the reduction in hydraulic conductance and increase in embolism in response to infection (McElrone et al. 2003). Similarly, *Senecio vulgaris* (groundsel) subjected to combined drought and *Puccinia lagenophorae* (causal agent of rust) infection had decreased leaf water potential as compared to plants under individual stresses. In this study, reduction in leaf water potential was attributed to the cuticle rupture instigated by infection and its subsequent sporulation (Paul and Ayres 1984). In contrast, *Medicago sativa* (alfalfa) subjected to combined drought and *Verticillium albo-atrum* (causal agent of wilt) infection had high leaf water potential as compared to drought-stressed plants (Pennypacker et al. 1991). These studies indicate that effect on leaf water potential varies with the type of plant and pathogen. QTL linked with leaf water potential was demonstrated in rice plants under drought stress (Table 4.1). Nonetheless, characterization of QTL under combined stress is yet to be demonstrated.

4.2.3 Stomatal Movement

Plants alter stomatal movement (closure or opening) in response to both drought and pathogen infection to impart tolerance (Ramos and Volin 1987; McElrone et al. 2003; Melotto et al. 2006). *Vitis vinifera* (grapevine) plants subjected to drought

stress had less stomatal conductance than that of well-watered plants. Drought severity was negatively correlated with the stomatal conductance of plants (Tombesi et al. 2015). *Solanum lycopersicon* (tomato) with less stomatal frequency infected with *Xanthomonas campestris* (causal agent of bacterial spot disease) had less infection than the plants with high stomatal frequency. In addition, plants treated with antitranspirant (phenylmercuric acetate) or abscisic acid (ABA) had less infection under spray inoculation as compared to the control plants. Further, plants subjected to spray inoculation had less infection as compared to plants infected with infiltration mode of inoculation. Here, reduction in infection in spray inoculated plants was attributed to the stomatal closure in response to infection (Ramos and Volin 1987). Similarly, *Arabidopsis* plants infected with *P. syringae* had no infection at the initial hours as compared to mock-inoculated plants. However, infection was observed in the later hours, and the reason behind such plant response was pathogen-induced stomatal opening. The resistance observed during the initial period of infection was conferred by the stomatal closure (Melotto et al. 2006). Stomatal response under combined stress has been recently investigated. Grapevine subjected to combined drought and *X. fastidiosa* infection had low stomatal conductance as compared to plants under individual stresses. Reduction in stomatal conductance under combined stress was additive as compared to plants subjected to individual stresses. The additive effect was attributed to drought caused a reduction in hydraulic conductance and infection instigated vessel occlusion (McElrone et al. 2003; Choi et al. 2013). These studies indicate that stomatal frequency and stomatal conductance play a role in individual and combined stress in imparting tolerance. A hypothetical model depicting the primary underlying mechanism of stomatal movement under individual and combined drought and pathogen infection is provided (Fig. 4.1). QTLs linked with the stomatal movement were reported in wheat (Table 4.1). However, under combined stress, the role of these QTLs is yet to be studied.

4.2.4 Root Length

Change in primary root length and lateral root number has been shown as a plant response to both individually occurring drought and pathogen infection and their combination (Blaker and MacDonald 1981; Dryden and Van Alfen 1984). Maize recombinant inbred lines (RIL) with fewer lateral roots but longer primary root length showed better performance as high leaf relative water content was demonstrated under drought stress than RIL with more lateral roots and shorter primary root length (Zhan et al. 2015; Gao and Lynch 2016). Oren et al. (2003) demonstrated that lateral roots served as the site of initial infection of *F. verticillioides* (causal agent of rot and wilt) in maize, whereas primary root was free of infection. Similarly, when maize plants were subjected to *F. verticillioides* infection, lateral roots were the vulnerable site of infection irrespective of resistant or susceptible genotype (Wu et al. 2013). These studies indicate that increase in lateral roots play a negative role in plant response to drought and pathogen infection as it did not aid in water absorption and increased the infection, respectively. Similarly, the role of

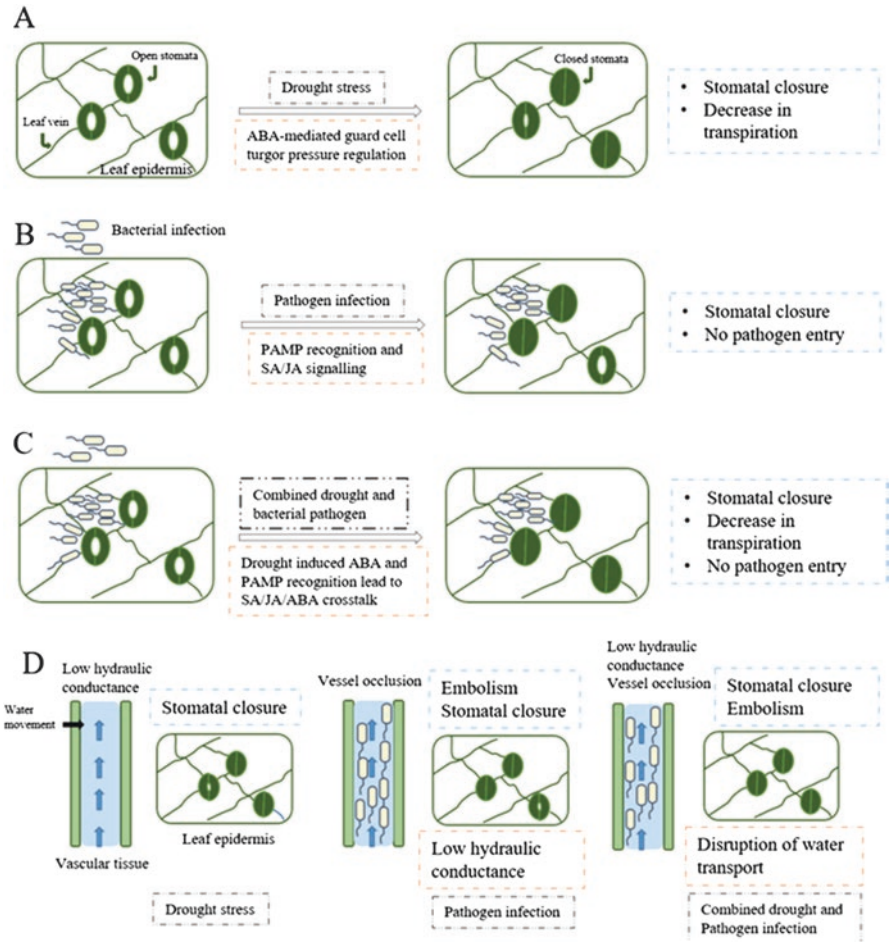

Fig. 4.1 Hypothetical model depicting the role of stomatal movement under combined drought and pathogen to impart stress tolerance. (**a**) Under drought stress, *A. thaliana* induces 9-cis-epoxycarotenoid dioxygenase (NCED3), which promotes ABA biosynthesis, and it regulates stomatal movement to avoid water loss (Iuchi et al. 2001). (**b**) *A. thaliana* activates salicylic acid (SA)/jasmonic acid (JA) as a plant response to pathogen-associated molecular pattern (PAMP) from foliar pathogen (e.g., *Pseudomonas syringae*) and regulates stomatal movement (Melotto et al. 2006). (**c**) Under combined drought and foliar bacterial pathogen infection, *A. thaliana* could upregulate ABA level, which could then interact with SA/JA and tune stomatal movement. This event is yet to be fully understood. (**d**) Under drought stress, *Parthenocissus quinquefolia* (vine) closes stomata, while under *Xylella fastidiosa* (vascular bacterial pathogen) infection, plant induces embolism as well as stomatal closure. Under combined stress, plant induces stomatal closure to prevent water loss. Additionally, embolism is induced as plant experiences low hydraulic conductance and vessel occlusion (McElrone et al. 2003; Choi et al. 2013). Blue box represents plant response to stress; orange box represents stress impact on plants; gray box represents stress imposed on plants

primary root length has been studied under combined stress. Dryden and Van Alfen (1984) demonstrated that under combined drought and *F. solani* f. sp. *phaseoli* (causal agent of root rot) infection, lateral bean roots present at topsoil were infected more, whereas primary roots were infection-free. This susceptibility was ascribed to the variation in pathogen concentration along the soil depth, and plants with long primary root showed tolerance to the combined stress (Dryden and Van Alfen 1984). Cotton plants with fewer lateral roots experienced less infection than a plant with more lateral roots which sustained higher infection. Additionally, plants with increased primary root length manifested better plant growth under combined drought stress and *V. albo-atrum* infection as it had access to water present at greater soil depth. Moreover, the plants had escaped from the infection, which was prominent at topsoil (Cook and Papendick 1972). We conclude that increased primary root length and reduction in lateral root number might attribute to the tolerance of plants under combined stress. However, the role of primary root length in conferring tolerance to combined stress varies with the type of plants and their age. Additionally, QTL associated with root length was determined in wheat crops under individual drought stress (Table 4.1). However, the effect of QTL under combined stress is yet to be elucidated.

4.3 Impact of Combined Stress on Other Morpho–Physiological Parameters

4.3.1 Leaf Area

Plants respond to drought stress and pathogen infection by altering leaf area (Lopez et al. 1997; Williams and Ayres 1981; McElrone et al. 2001; Bradley et al. 2003). Under drought stress, *Cajanus cajan* (pigeon pea) had decreased leaf area, which resulted in low yield as compared to well-watered plants (Lopez et al. 1997). Bradley et al. (2003) demonstrated that under pathogen stress, leaf area of *Trifolium repens* (clover) was negatively correlated with susceptibility to *Stemphylium* spp. (causal agent of Stemphylium leaf spot) infection. Under combined drought and *Erysiphe graminis* infection (causal agent of powdery mildew), *Hordeum vulgare* (barley) had altered its leaf area in response to drought but not to pathogen infection. No difference was observed in transpiration rate and photosynthesis in plants subjected to combined stress and individual pathogen infection (Williams and Ayres 1981). Similarly, under combined drought and *X. fastidiosa* infection on Virginia creeper, a significant reduction in leaf area was demonstrated, while infection alone did not affect the leaf area (McElrone et al. 2001). These studies demonstrate that under combined stress condition, infection did not contribute to the plant susceptibility. On the other hand, groundsel subjected to combined drought and *P. lagenophorae* infection had reduced leaf area, and reduction in leaf areas was aggravated in an additive manner under combined stress (Paul and Ayres 1984).

Similarly, bean plants exposed to combined drought and *Macrophomina phaseolina* (causal agent of charcoal rot) infection had reduced leaf area as compared to plants subjected to infection alone (Mayek-PÉrez et al. 2002). As a consequence, photosynthesis was compromised more in combined stressed plants as compared to infected plants. These studies indicate that under combined stress, the effect of drought on leaf area resulted in a reduction in infection.

4.3.2 Leaf Greenness

As a plant response, change in chlorophyll content has been observed under combined drought and pathogen infection (Gupta et al. 2016; Sinha et al. 2016), and this can be associated with leaf greenness. Leaf greenness was employed as a parameter to assess drought stress effect in *S. tuberosum* (potato) and grapevine (Fanizza et al. 1991). Potato plants subjected to drought stress showed increased leaf greenness as compared to the well-watered plants, and the increment in greenness was accompanied by a reduction in leaf growth and delayed leaf senescence (Rolando et al. 2015). In contrast, *Saccharum officinarum* (sugar cane) infected with *P. kuehnii* (causal agent of orange rust) display decreased SPAD index, an indicator of chlorophyll content, and low photosynthetic rate (Zhao et al. 2011). These studies indicate that drought stress induced the increase in chlorophyll content, which was associated with tolerance, whereas pathogen infection reduced it, which led to susceptibility. Similarly, the effect of combined stress has been investigated on chlorophyll content. For example, chickpea plants subjected to combined drought and *Ralstonia solanacearum* (causal agent of wilt) infection had high chlorophyll content as compared to the infected plants. Additionally, infection level was found less in plants under combined stress as compared to plants subjected to infection alone and which was associated with tolerance under combined stress (Sinha et al. 2016). Similarly, chickpea plants subjected to combined drought and *P. syringae* infection had more chlorophyll content than the plants under infection alone. Further, low infection level was demonstrated in plants under combined stress as compared to the plants under infection alone (Sinha et al. 2016). Similarly, *Arabidopsis* plants subjected to combined drought and *P. syringae* infection had increased chlorophyll content as compared to only infected plants (Gupta et al. 2016). These studies specify that despite an increase in chlorophyll content which was observed under combined stress, its role in imparting tolerance is yet to be explained.

4.3.3 Canopy Temperature

Plants display altered transpiration that changes canopy temperature in response to drought stress and pathogen infection to sustain growth. Canopy temperature has been used to determine plant water status in many crops (Stark et al. 1991; González-Dugo et al. 2006). Wheat plants subjected to drought stress showed higher canopy

temperature as compared to the well-watered plants. It was found that plants with higher canopy temperature were susceptible to drought stress, whereas plants with less canopy temperature were tolerant (Blum et al. 1989). Wheat plants showed high canopy temperature when infected with *Mycosphaerella graminicola* (causal agent of tritici blotch) as compared to non-infected plants. The canopy temperature was positively correlated with disease severity (Eyal and Blum 1989). Similarly, *Cucumis sativus* (cucumber) infected with *Pseudoperonospora cubensis* (causal agent of downy mildew) pathogen had higher canopy temperature as compared to non-infected plants (Oerke et al. 2006). Similarly, the impact of combined stress on canopy temperature has been studied. For example, wheat plants subjected to combined drought and *P. striiformis* (causal agent of stripe rust) infection had high canopy temperature as compared to pathogen-only infected plants (Smith et al. 1986). Similarly, *Beta vulgaris* (beet) under combined drought and *Pythium aphanidermatum* (causal agent of root rot) infection manifested increased canopy temperature as compared to only infected plants (Pinter et al. 1979). Likewise, under combined drought and *Phymatotrichum omnivorum* infection, cotton plants had high canopy temperature as compared to only infected plants. Also, plants moderately infected did not show wilting under drought stress (Pinter et al. 1979). These studies indicate that changes in canopy temperature influence combined stress tolerance.

4.3.4 Time to Anthesis

Plants undergo changes in anthesis time and post-anthesis developments such as grain filling that influence grain number and size under drought stress and pathogen infection (Mahalakshmi and Bidinger 1985; Manjarrez-Sandoval et al. 1989; Winkel et al. 1997). *Sorghum bicolor* (sorghum) plants subjected to drought stress before and after anthesis had less grain number and reduced grain size, respectively, as compared to the well-watered plants. This effect was attributed to the delay in anthesis (Manjarrez-Sandoval et al. 1989). Similarly, *Pennisetum glaucum* (pearl millet) subjected to drought stress before anthesis had less grain number as compared to the control plants (Mahalakshmi and Bidinger 1985; Winkel et al. 1997). In contrast, *Arabidopsis* plants infected with *P. syringae* before anthesis showed accelerated anthesis as compared to the mock-treated plants. Also, infection increased the basal branches, which resulted in more number of primary inflorescence (Korves and Bergelson 2003). Likewise, wheat plants were more susceptible to *Gibberella zeae* (causal agent of Fusarium head blight) infection during the time of anthesis. This susceptibility was ascribed to the presence of partially exserted anthers. Plants with fully exserted anthers had dehisced along with inoculum as compared to the plants with partially exserted anthers, which acted as a source of inoculum (Reis et al. 2016). Under combined drought and *Verticillium albo-atrum* (causal agent of wilt) infection, Medicago plants had experienced a delay in anthesis compared to drought-stressed plants (Pennypacker et al. 1991). These studies imply that anthesis is affected by both individual and combined stress conditions.

4.4 More Potential Traits: Analysis from Individual Stress Studies

4.4.1 Trichome Type and Density

Plants alter trichome number in response to drought or pathogen infection (Ehleringer et al. 1976; Wagner 1991; Wagner et al. 2004). *Cbp20* (cap binding protein 20) mutant of *Arabidopsis* was shown to be drought tolerant. Mutant plants subjected to drought stress had more trichomes and low stomatal conductance as compared to the wild-type plant (Papp et al. 2004; Jäger et al. 2011). *Phlomis fruticosa* (Jerusalem sage) and *Hedera helix* (ivy) with non-glandular trichomes had entrapped dew and facilitated water absorption under drought stress as compared to plants without trichomes (Grammatikopoulos and Manetas 1994). Glandular trichomes of *Nicotiana tabbacum* (tobacco) infected with *Peronospora tabacina* (causal agent of blue mold disease) secreted potent inhibitors, namely, T-phylloplanins, which inhibited the infection, as compared to mock-inoculated plants. In addition, enrichment of T-phylloplanins secreted by glandular trichomes was observed with increase in trichome number (Kroumova et al. 2007; Nguyen et al. 2016). Similarly, glandular trichomes of potato infected with *Phytophthora infestans* (causal agent of late blight) secreted oxidative enzymes, which reduced pathogen infection as compared to plants without trichome (Lai et al. 2000). Barley plants subjected to drought and pathogen infection had increased non-glandular trichome number as compared to control plants. However, fungal biomass was increased with trichome number under pathogen infection as compared to mock-inoculated plants (Liu and Liu 2016). These studies indicate that tolerance is associated with trichome number and the type of trichome. Glandular and non-glandular trichomes serve as a physical defense against herbivory as they synthesize and secrete antimicrobial molecules (Ehleringer et al. 1976).However,under combined stress, the significance of trichome number is yet to be understood. It is evident that plants which possess both glandular and non-glandular trichomes might resist drought as well as pathogen infection by absorbing water and resisting infection. So it is noteworthy to study trichome as a trait under combined stress.

4.4.2 Cuticular Wax Composition

Plants alter cuticular wax composition in response to drought and pathogen infection (Marcell and Beattie 2002; Kosma et al. 2009). *Sesamum indicum* (sesame) subjected to drought stress had a high density of cuticular wax as compared to well-watered plants (Kim et al. 2007). Similarly, *Arabidopsis* plants subjected to drought had increased cuticular wax deposition as compared to well-watered plants (Kosma et al. 2009). Glossy mutants of maize plants infected with *Clavibacter michiganensis* (causal agent of Goss's wilt) pathogen retained more bacteria on its leaf surface, whereas wild-type plants had retained fewer bacteria and resisted infection. Susceptibility in the glossy mutant was attributed to the reduced density of cuticular

wax crystals as compared to the wild-type plants (Marcell and Beattie 2002). A bloomless mutant of sorghum was used to study water loss and susceptibility to *Setosphaeria turcica* (causal agent of leaf blight) infection. Bloomless mutants had lost more water as compared to wild-type plants. In addition, bloomless mutants sustained more infection as compared to wild-type. Increased water loss and susceptibility to infection were ascribed to the reduced cuticular wax deposition in mutants (Jenks et al. 1994). This evidence indicates the cuticular wax composition play a role in drought and disease susceptibility to drought and pathogen infection. However, the significance of cuticular wax under combined stress is yet to be studied.

4.5 Molecular Mechanism Governing Traits Imparting Combined Stress Tolerance in Plants

Recently, several molecular and physiological studies have been undertaken to comprehend the gene expression patterns associated with tolerance to combined drought and pathogen infection. Marked change in expression of genes between the plants subjected to individual and combined stress was observed (Rizhsky et al. 2004; Prasch and Sonnewald 2013; Rasmussen et al. 2013). Majorly, under combined stress, gene expression pattern was categorized into two major divisions, viz., unique (idiosyncratic) and tailored or shared. Under combined stress, genes which are expressed uniquely are named as tailored, whereas genes which are expressed in both individual and combined stress conditions are termed as shared or common (Pandey et al. 2015; Gupta et al. 2016). Molecular basis of some of the morpho-physiological traits associated with tolerance in response to combined stress has been studied. For instance, stomatal behavior under combined drought and pathogen stress may result in either plant tolerance or susceptibility. A study conducted by Mantri et al. (2010) reported the transcriptional changes in *A. rabiei*-infected chickpea as compared to control. In this study, it was found that the genes involved in metabolism and photosynthesis were suppressed under individual drought stress treatment. Importantly, pathogenesis-related proteins were repressed under drought stress. In contrast, under pathogen stress, pathogenesis-related proteins were induced in chickpea. In *A. thaliana*, Gupta et al. (2016) reported that the genes such as *NTM1* (NAC with transmembrane motif1 (NTM1) and *PNP* (plant natriuretic peptide) were upregulated only under combined drought and *P. syringae* infection but not under individual stress conditions. Importantly, PNP protein has been reported to be involved in regulation of stomatal conductance (Turek et al. 2014). In addition, genes encoding JAZ proteins were also upregulated under combined drought and pathogen stress but downregulated in individual pathogen stress (Gupta et al. 2016). JAZ-interacting domains are found in bHLH protein family, which has been reported to be involved in trichome development and stomatal patterning (Pauwels and Goossens 2011). Silencing of genes encoding JAZ proteins in *S. lycopersicum* resulted in rapid disease development and also delayed the PAMP-mediated hypersensitive reaction upon *P. Syringae* infection as compared to control (Ishiga et al. 2013). Similarly, *Arabidopsis* mutants such as *ssi2* (stearoyl desaturase) and *fad7/fad8* (fatty acid desaturase 7 and 8) were demonstrated in defense manifesting

resistance to oomycetes and *P. Syringae* infections. Genes governing lipid metabolism were also upregulated in drought-stressed rice as compared to control. Cell membrane stability is regulated by lipid content of the plants. Therefore change in lipid content or alteration in lipid metabolism results in plant tolerance (Borah et al. 2017; Rojas et al. 2014). Identification and functional characterization of genes governing morpho-physiological traits will assist in plant tolerance to combined stresses.

4.6 Future Perspectives

Crop plants alter morpho-physiological features to survive in the stressful environment. In plant breeding, researchers exploited morpho-physiological response-based traits such as root length for crop improvement under individual drought stress. However, as plants encounter simultaneous stresses in field conditions, for example, combined drought and pathogen infection, studies should be undertaken to evaluate the extent of the contribution of several of already known traits to combined stress tolerance. Additionally, understanding of the underlying molecular mechanism of the known traits, with reference to combined stress, is important. Although studies have shown the changes in traits such as cell membrane stability, leaf water potential, stomatal conductance, and root length in response to combined stress, a direct correlation about the contribution of these traits to combined stress tolerance is yet to be studied. Additionally, few papers have pointed out the plausible mechanisms such as drought-activated hydraulic signals, which alter stomatal movement under drought stress (Yan et al. 2017), exogenous and endogenous ABA-mediated changes in stomatal movement under drought and pathogen infection (Mohr and Cahill 2003), and drought-induced ROS-mediated resistance to bacterial infection which was demonstrated in *N. benthamiana* (Ramegowda et al. 2013). In a nutshell, it is essential to undertake a systematic investigation to study the molecular mechanism responsible for tolerance or susceptibility of plants to combined stress.

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Chapter 5
Genome Editing for Crop Improvement: Status and Prospects

Pooja Manchanda and Yadhu Suneja

Abstract Genome editing using sequence-specific nucleases has been one of the fast-evolving technologies ever since the discovery of CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated endonuclease) system. CRISPR locus present in the genomes of various bacteria and archaea offer a programmable defense mechanism against foreign agents like viruses. The understanding of this biological phenomenon leads to the development of genome editing tool for making specific modifications in the genomes of various organisms. The CRISPR/Cas9 technology basically depends upon two components: Cas9 enzyme and sgRNA (single-guide RNA). These two components are delivered into plant cells using different gene transfer methods such as *Agrobacterium*-mediated, biolistic (particle gun) approach, pre-assembled ribonucleoproteins followed by homology-based detection of target DNA by sgRNA, DNA cleavage by Cas9, and DNA repair by native cell machinery employing nonhomologous end joining. This usually leads to a frameshift and knockout mutation in the targeted gene. Providing a template DNA for homology-dependent repair can extend this technique to knock in mutations as well. This technology thus opens up a unique opportunity for directed alterations in chosen genes. Hence, CRISPR/Cas technology has huge potential as a precise and rapid plant breeding method. The absence of foreign DNA introduction (particularly in the case of gene knockout) is anticipated to attract fewer biosafety concerns as compared to GMOs in the regulatory frameworks coming up in different countries. This chapter reviews the CRISPR-Cas strategy by focusing on components of the tool kit and available variants, delivery into plant cells, and gene modification detection assays. A set of crop improvement-related studies, targeting genes of basic as well as applied significance, are listed to illustrate its current use.

Keywords Genome editing · CRISPR/Cas9 · Single-guide RNA · Allelic variants

P. Manchanda (✉)
School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, India

Y. Suneja
Department of Biochemistry, Punjab Agricultural University, Ludhiana, India

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5.1 Introduction

Creation of genetic variation is the key element for crop improvement. As the world population is increasing, food security has become a challenging task with respect to limited land available for crop production (Ma et al. 2018). During the twentieth century, plant breeding has seen a tremendous change fueled by our increasing knowledge of genetic phenomena. Modern plant breeding is entering a new era with the emergence of various sequence-specific nucleases (SSNs). SSNs mediate efficient editing of genomes of various mammalian and plant species. These SSNs induce double-strand breaks (DSBs) at specific chromosomal sites followed by their repair through nonhomologous end-joining (NHEJ) pathway resulting in nucleotide insertions and deletions. If homologous donor templates are available at the site of DSB, homology-directed repair (HDR) can also occur (Symington and Gautier 2011). The different types of SSNs are briefly described below:

5.1.1 Zinc-Finger Nucleases (ZFNs)

ZFNs are the hybrid proteins (nuclease domain is fused with DNA-binding domain) which provided a breakthrough for the manipulation of genomes of various crop species. ZFNs are regarded as the first generation (Bibikova et al. 2002) of the hybrid proteins to be used for genome editing purpose.

ZFNs are composed of zinc fingers which carry 30-amino-acid-long Cys_2-His_2 motif (folded into a ββα configuration and stabilized by a Zn^{2+} ion) constituting the DNA recognition and binding domain (Beerli and Barbas 2002). Each zinc-finger unit consists of three to four binding modules where each module recognizes a triplet nucleotide by inserting its α-helix into the major groove of the DNA double helix. Nuclease domain of Fok1 endonuclease (a type II restriction enzyme) when joined as a dimer to this zinc-finger unit further directs the cleavage at the identified site causing a double-strand break (DSB) (Carroll 2011; Weinthal et al. 2010). As tandem arrays of fingers can recognize extended contiguous sequences, at least three consecutive fingers are needed to provide an adequate binding affinity. As 18 bp of DNA sequence can confer specificity within billions of base pairs of DNA, designing arrays of six to eight ZFs with characterized recognition sites allows specific sequences to be targeted in the complex genome of different plant species. Since the availability of zinc-finger proteins as a site-specific nuclease to cleave target sites in the DNA, for many years, ZF protein technology was the only approach available to create custom site-specific DNA-binding protein and enzymes. Successful use of ZFNs for gene editing in model plants, *Arabidopsis* (Lloyd et al. 2005) and tobacco (Wright et al. 2005), was first reported in 2005, followed by its use for highly efficient gene targeting in tobacco (Townsend et al. 2009) and maize (Shukla et al. 2009). Several methodologies like “modular assembly” approach (Segal et al. 1999) and selection-based oligomerized pool engineering (OPEN)/

context-dependent assembly (CoDA) (Maeder et al. 2008) have been developed for assembling longer arrays and constructing zinc fingers. Companies like Gandaq Ltd. and Sangamo BioSciences (Richmond, CA, USA) have eased the use of ZFs by accumulating an extensive proprietary inventory of ZFs and ZF pairs and making them commercially available. The complex interactions between each amino acid in zinc-finger module with each base pair of target limit the designing of zinc-finger modules (Kumar and Jain 2015).

5.1.2 Transcription Activator-Like Effector Nucleases (TALENs)

In TALENs, the cleavage domain of Fok1 endonuclease is fused in-frame to TALE protein (transcription activator-like effector), a major virulence factor secreted by the phytopathogenic bacteria of the genus *Xanthomonas* that causes disease in plants by activating transcription of a specific target gene (Bogdanove and Voytas 2011; Weber et al. 2011). The value of these proteins for genome engineering was realized in 2009, when the TALE-DNA-binding code was discovered (Boch et al. 2009; Moscou and Bogdanove 2009). Each TALE protein is composed of variable copies of 33–35 amino acid repeats with each amino acid repeat carrying repeat variable di-residue (RVDR) at positions 12 and 13, respectively, which determines pairing with a specific base of target DNA sequence (Streubel et al. 2012). Different methods like fast ligation-based automatable solid-phase high-throughput (FLASH) and golden gate cloning have been developed to assemble target-specific repeat arrays in correct order. This potential led the journal *Nature Methods* to declare targetable nucleases as "The method of the year 2011" (Baker 2012), and TALENs also constitute second generation of SSNs (Boch et al. 2009). Apart from the custom TALEN construction facility offered by different companies like Life Technologies, Cellectis Bioresearch, ToolGen, CoWin Biotech, and Transposagen Biopharmaceuticals, TALEN core facilities are also available at different academic institutes like University of California, San Francisco, University of Utah, and University of Wisconsin, Madison. TALENs have been used for genome editing in a number of crops like rice, tobacco, wheat, etc. (Shan et al. 2013 and Wang et al. 2014).

5.1.3 CRISPR/Cas System

CRISPR (clustered regularly interspaced short palindromic repeats) and Cas (CRISPR-associated enzyme) are the latest addition to the toolbox of genome engineering. It is a more versatile genome editing platform developed in the recent years (Wang et al. 2016b). Among the various genome editing approaches (ZFNs, TALENs, and CRISPR) (Beumer et al. 2008; Cui et al. 2011; Bassett et al. 2013),

the CRISPR/Cas system is the method of choice because unlike ZFNs and TALENs, it does not involve intensive protein engineering (Mali et al. 2013; Gaj et al. 2013; Johnson et al. 2015) and requires only 22-nucleotide gRNA sequence for site-specific editing. Type II CRISPR/Cas system from *Streptococcus pyogenes* (Jinek et al. 2012) is an adaptive immune system in prokaryotes and provides defense against various viruses (Ishino et al. 1987) by degradation of foreign DNA in sequence-specific manner. This defense is acquired by integration of short fragments (spacers) of foreign agent between two adjacent repeats at the proximal end of CRISPR locus. In 2002, these "tandem repeats" were called "clustered regularly interspaced short palindromic repeats" (CRISPR) (Jansen et al. 2002; Horvath and Barrangou 2010). In 2005, the CRISPR spacer sequence was found to be highly homologous with exogenous sequences from bacterial plasmids and phages (Mojica et al. 2005; Pourcel et al. 2005). Due to the presence of homology between host and foreign DNA, CRISPR is able to cleave foreign DNA resulting in the development of CRISPR/Cas system as sequence-specific genome editing tool (Doudna and Charpentier 2014). During subsequent attack by viral/foreign DNA, the CRISPR arrays, including the spacers, are transcribed and are processed into small CRISPR RNAs (crRNAs), approximately 40 nt in length, which combine with trans-activating CRISPR RNAs (crRNAs) to activate and guide Cas9 nuclease (Barrangou et al. 2007), resulting in the cleavage of homologous double-stranded DNA sequences (protospacers) of foreign DNA. An essential requirement for cleavage is the presence of protospacer adjacent motif (PAM), i.e., NGG, downstream of target DNA (Jinek et al. 2012) but less frequently NAG (Hsu et al. 2013). The region presenting 12 base pairs upstream of PAM is the seed region, which must match between target DNA and RNA. This three-component system of crRNA, tracrRNA, and Cas protein was reduced to two-component system when the targeting specificity of crRNA was combined with structural properties of tracr in a chimeric sgRNA (Jinek et al. 2012). CRISPR/Cas system is an example of RNA-guided endonucleases (RGENs) which are based on the RNA (and not protein)-guided mode of DNA recognition. Ever since the first report published in 2013, stating the deployment of CRISPR/Cas system for gene targeting in mammalian cells appeared, extensive studies have followed up to explore the potential of this game-changing technology to target different traits in diverse crop species.

Unlike ZFs and TALENs, site-specific catalytic action of sgRNA-Cas9 complex is governed by the sequence of only 20 consecutive nucleotides that constitute chimeric gRNA. Accordingly, designing gRNA corresponding to the target DNA sequence and synthesis of this oligonucleotide is far simpler. Additionally, Cas9 nuclease does not require reengineering for each new target site. Shorter length of sgRNA sequence allows a more convenient delivery into the cells than the long and highly repetitive ZFN-/TALEN-encoding vectors. Off-targets due to non-specific binding of zinc-finger motifs have also been reported (Weinthal et al. 2010; Voytas 2013). Multiplexing sgRNA corresponding to different genes and simultaneous delivery/co-transformation is far more easily achievable relative to TALENs/ZFNs.

5.2 Components of CRISPR/Cas System as an Efficient Genome Editing Tool

To cleave the target DNA, Cas9 derived from *S. pyogenes* (*SpCas9*) recognizes PAM motif in the target site (Mojica et al. 2009), binds the target sequence as recognized by sgRNA which pairs with 19–22 bases complementary to DNA sequence upstream of the PAM, and cleaves the target DNA (Jinek et al. 2012). Different components of the CRISPR-Cas9 system are briefly discussed below:

5.2.1 Cas9 Enzyme (CRISPR-Associated Endonuclease)

Cas9 nuclease is maintained in an auto-inhibited conformation as observed during single-particle electron microscopy, and it becomes active only after gRNA gets loaded into it (Jinek et al. 2014). Upon recognition of PAM sequence, it further undergoes conformational change to form a central channel for RNA-DNA heteroduplex binding (Anders et al. 2014). Cas9 is a bilobed structure consisting of two major lobes, an N-terminal large REC lobe (recognition lobe) and a C-terminal small NUC lobe (nuclease lobe) (Nishimasu et al. 2014). REC lobe does not share structural similarity with other known proteins, indicating that it is a Cas9-specific functional domain. REC lobe further consists of three domains, i.e., α-helical segment, RuvC domain, and bridge helix domain. α-Helical segment consists of two sub-domains (RecI and RecII), out of which RecI is the largest sub-domain and is responsible for binding of gRNA by Watson-Crick base-pairing. The function of RecII domain is still unclear. Bridge helix domain is arginine rich and is responsible for linking REC lobe to NUC lobe. NUC lobe also possesses three domains, namely, helix-nuclease-helix (HNH) domain, RuvC endonuclease domain, and PAM-interacting (PI) domain. Helix-nuclease-helix is a nuclease domain which cleaves the DNA strand complementary to gRNA, three nucleotides upstream of PAM sequence. RuvC endonuclease is another nuclease domain which cleaves the DNA strand not complementary to gRNA. PAM-interacting (PI) domain is responsible for recognition of protospacer adjacent motif (PAM) on noncomplementary strand of DNA.

There are varieties of Cas nucleases available today, which can be utilized for various applications like high-throughput genome editing, knocking out the expression of gene, and transcriptional control with improved specificity and reduced off-target effects in various systems, ranging from fruit fly, yeast, bacteria, plants, etc. (Khatodia et al. 2016). Different variants of Cas9 nuclease are explained as follows:

5.2.1.1 *SpCas9*

SpCas9 is a wild-type or native Cas9, derived from *Streptococcus pyogenes* (size, 900–1600 amino acids). It cleaves dsDNA at a specific site resulting in the creating of double-strand break repair machinery, resulting in dsDNA cleavage, further activating nonhomologous end joining (NHEJ). If a donor template having homology to target locus is present, homology-directed pathway for repair (HDR) can also be followed for the introduction of specific changes (Hsu et al. 2013). It recognizes NGG as PAM sequence. It is successfully used in plant and mammalian cells.

5.2.1.2 Inactive Cas9 or dCas9

dCas9 is a nuclease-deficient but DNA-binding enzyme formed by the induction of mutations in H840A subunit in HNH domain and D10A subunit in RuvC domain (Qi et al. 2013). It is used for overexpressing (CRISPRa) or silencing of gene (CRISPRi) by fusion with effector domains. It is used as a visualization tool for the detection of repetitive and non-repetitive loci. dCas9 tagged with an affinity is being used for studying protein-genome interactions through chromatin immunoprecipitation assays (Fujita and Fujii 2014).

5.2.1.3 *SaCas9*

SaCas9 is derived from *Staphylococcus aureus*. It functions similar to *SpCas9* but recognizes NNGRRT (where R = A or G) as PAM sequence. The size of *SaCas9* is relatively smaller (1053 amino acids), which makes the process of delivery relatively easier (Ran et al. 2013). It is successfully used in mammalian cells.

5.2.1.4 *Cas9^{D10A}*

Cas9^{D10A}is formed by the induction of mutations in D10A subunit of *SpCas9* (Shen et al. 2014). It cuts only one strand of DNA, thereby, generating single-strand breaks, which can be repaired and no indels are produced. This enzyme is used for minimizing off-target effects by employing double-nicking strategy with paired nickases targeting adjacent regions.

5.2.2 Target Site Identification and gRNA Designing

sgRNA sequence is composed of a constant tracrRNA and variable crRNA (Jinek et al. 2012). Within sgRNA, the guide sequence has an established length of 20 nucleotides followed by a three-base PAM sequence at its 3′ end. The potential of

CRISPR-Cas9 system to target a specific sequence may be limited by the availability of PAM sites (Wei et al. 2015). The selection of a suitable target site is one of the most important determinants of efficiency of Cas9 and induction of on-target mutations. Aside from the manual selection of sgRNA target site, several web-based tools are available for genome-wide prediction of highly specific sgRNA and detection of off-targets in model plants and major crops (Bae et al. 2014; Heigwer et al. 2014; Xiao et al. 2014). Any undesired change or off-target cleavage could lead to cytotoxicity, apoptosis, and gross chromosomal rearrangements like inversion, deletion, or translocations (Lee et al. 2012; Park et al. 2014; Hendel et al. 2015).

sgRNA on-target cleavage efficiency is determined by DNA sequence profile, accessibility of the gene locus (packaging of chromosome), and nucleotide composition of DNA downstream of the spacer region. Different softwares provide unique scores for the activity (Cas9 on-target efficiency) and specificity (off-target prediction accuracy) of the target site cleavage by Cas9 and accordingly rank the suggested sgRNA sequences by their efficiency and specificity (Bolukbasi et al. 2016). Based on the mode of delivery chosen for CRISPR reagent, separate scores may be used, for example, for U6 promoter-based assays, Fusi/Doench score (Doench et al. 2016) and, for assays based on direct delivery of gRNA produced by T7-based IVT, Moreno-Mateos score may be used (Haeussler et al. 2016). For a large set of possible guides to pick from an additional ranking criteria, Wong score for U6 promoter-based guides (Wong et al. 2015) or GG rule (for T7-based IVT) may be followed. The softwares also calculate and provide a score for off-target mutation based on the distance of the mismatch to the PAM—Hsu Zhang score, MIT score (all potential off-targets may be summarized into guide specificity score that ranges from 0 to 100), and CFD (cutting frequency determination) score that distinguishes between validated and false-positive off-targets (Doench et al. 2016). Off-target scores are generally identified and reported for exonic regions (and not intergenic regions, introns, or UTRs). Further these softwares may provide information about restriction enzyme site within the predicted gRNA, primer sequence for cloning gRNA into vector and detecting on-target mutations (Periwal 2017). A few of the sgRNA design tools like CCTOP, COSMID, CRISPR optimal, Target Finder, and SAPTA have shown experimental validation of their prediction results, thus substantiating their effectiveness. Others like CROP-IT sgRNA scorer predict sgRNA based on the information obtained from previously tested engineered nucleases.

There are certain points that must be kept in mind while selecting the target site and designing the gRNA for CRISPR-Cas experiment. To select a unique target sequence and minimize chances of off-target cleavage, a BLAST search of the whole genome sequence of the target specific for the relevant 22-nucleotide sequence may be performed (Chuai et al. 2016). CRISPR-Cas9 system discriminates efficiently against any potential off-target sites with perfect match in the 12-nucleotide-long PAM-proximal sequence (seed sequence), while mismatches in the PAM-distal sequence are actually tolerated. sgRNA with two extra target-independent GG nucleotides at the 5′ terminus have been found to confer significant specificity to the Cas9 action when compared with conventional sgRNA (Cho et al. 2014). Target sequence at the 3′ end of the coding region or intron should be avoided.

While searching, PAM sequence should be looked upon in the complementary genomic sequence as well. Quadruplex-forming homopolymer or a stretch of repeat sequence in the target gene should be avoided. GC content should be >30% but less than 80%. The chosen gRNA should not lead to the formation of a stable secondary structure in the protospacer. It should be designed from the proximal region, i.e., 5′ end of the gene. The functional domains of the gene of interest may be considered, but targeting highly conserved region of a gene family should be avoided. More than three mismatches (especially in the seed region) between DNA target and gRNA may not be tolerated.

5.3 Modes of Delivery of CRISPR Components into Plants

RNA-guided endonucleases (RGENs) were proclaimed as "Breakthrough of the year 2015" by the *Science* journal for its broader application to numerous disciplines of life sciences. CRISPR-Cas9 system superseded the potential of previous GE tools (MegaN, ZFNs, and TALENs) on account of its easier preparation, affordable nature, and flexible usage (Kim and Kim 2014). Because of the presence of a rigid plant cell wall, delivery of GE reagents into the plant cells is a major barrier to the use of this technology for creating novel traits (Baltes et al. 2014). Therefore, introduction of CRISPR/Cas construct into plant cells is carried out through various direct (vectorless) and indirect (vector-mediated) gene transfer methods. The various direct methods for delivery of CRISPR/Cas construct into plant cells are electroporation, PEG (polyethylene glycol)-mediated transfection, and particle gun method. The indirect methods involve the introduction of construct through a vector or intermediate such as bacteria (e.g., *Agrobacterium tumefaciens* or *A. rhizogenes*) or plant virus systems [e.g., tobacco rattle virus (TRV), lentivirus, geminivirus, etc.].

5.3.1 Direct or Vectorless Methods

5.3.1.1 Electroporation or PEG-Mediated Method

Electroporation (using electrical impulse) or chemical transformation (using PEG) is usually carried out for testing the efficacy of various constructs for transient assays using protoplasts obtained after digestion of cell with cell wall-degrading enzymes like cellulose and pectinase. It is usually carried out for the validation of gRNAs. Protoplasts isolated from different crop species (rice, wheat, maize, lettuce, tobacco, tomato, *Arabidopsis*, etc.) have been used to evaluate gene editing reagents using CRISPR/Cas9-based systems (Liang et al. 2014; Shan et al. 2014; Cermak et al. 2015). Protoplasts can be used to determine target site mutagenesis efficiency and can be regenerated into plants (Woo et al. 2015).

5.3.1.2 Particle Gun Method

Particle gun method, also known as biolistic method, is a method of choice for the direct delivery of CRISPR/Cas construct into target cells of many crop plants using gold/tungsten microcarriers and uses the physical force to transfer the construct into plant cells. Transgene-free genome-edited crop plants have been produced in maize (Svitashev et al. 2016) and hexaploid wheat (Liang et al. 2017) through biolistic approach.

5.3.2 Indirect or Vector-Mediated Methods

5.3.2.1 *Agrobacterium*-Mediated Genome Editing

Agrobacterium tumefaciens-mediated genetic transformation is one of the most suitable and efficient methods for the production of stable transgenic plants. A number of crop plants have been genome-edited in the past few years using *Agrobacterium tumefaciens.* It is carried out by infecting various explants like callus tissues in *Agrobacterium* broth followed by the regeneration of T_0 plants under in vitro conditions (Gao et al. 2015). *Agrobacterium*-mediated delivery of CRISPR/Cas9 construct into immature embryos of maize resulted in editing of various genes (Svitashev et al. 2016). But there are two major limitations of *Agrobacterium*-mediated genome editing: (i) it cannot be used for introducing ribonucleoprotein complex into target cells and (ii) there are greater chances of stable transformation leading to the integration of Ti plasmid backbone into the plant genome resulting in the production of transgenic plants.

5.3.2.2 Viral Vector-Mediated Genome Editing

Viruses are also used as vectors for the delivery of CRISPR/Cas constructs alone or along with donor DNA templates for carrying out programmable editing in plants. Viral vectors are one of the desired vectors for the introduction of genome editing reagents into target cells (Maggio and Goncalves 2015). They are also used for direct delivery of recombinant proteins into target cells after fusion to structural components of vector particles (Skipper and Mikkelson 2015). Lentiviral vectors are used for genetic modification of target cells due to integrase-dependent mechanisms which results in chromosomal insertion of transported foreign nucleic acids. However, for genome editing experiments, integrase mechanisms of lentivirus must be disabled to ensure episomal vector templates as substrates for homologous recombination or for transient designer nuclease expression. The first viral vector used for gene-silencing (VIGS) study was tobacco mosaic virus (TMV) (Kumagai et al. 1995). The process of inoculation becomes easier using geminiviruses (DNA viruses), since it requires only viral DNA. Geminiviruses have been modified for

homology-directed recombination-mediated genome targeting and achieving desired modifications in crop plants (Gil-Humanes et al. 2017). Virus-based guide RNA delivery system for CRISPR-Cas9-mediated genome editing (VIGE) using geminivirus was carried out for targeting endogenous *PDS3* (*phytoene desaturase*) and *IspH* (*isopentenyl/dimethylallyl diphosphate synthase*) genes, involved in non-mevalonate pathway of isoprenoid synthesis, using cabbage leaf curl virus because it can infect plants systemically resulting in albino phenotype of tobacco plants (Yin et al. 2015). TRV is also being used as a tool for genome editing because it has a bipartite genome consisting of two positive-sense single-guide RNAs, RNA1 and RNA2. RNA2 genome can be modified to carry exonic gene fragments (Kumar et al. 2003). Inoculation of virus is carried out through mechanical or agro-infiltration process. As there occurs no integration of viral genome into plant genome, the edited products are not transformed.

5.4 Detection of On-Target Modifications Following Genome Editing

The indel-forming nonhomologous end-joining (NHEJ) pathway repairs double-strand breaks of various crop plants under in vivo conditions (Jinek et al. 2013), resulting in mutation following genome editing. NHEJ is an error-prone DNA repair mechanism, which results in small insertions and/or deletions (indels) at the site of the break. As a result of indels, there occurs frameshift mutation and production of termination codon which can result in knockout of the function of the gene due to the production of truncated polypeptides (Perez et al. 2008; Santiago et al. 2008; Sung et al. 2013; Ramleet et al. 2015). Therefore, after the introduction of CRISPR/Cas construct or gRNA-Cas ribonucleoprotein complex into the genome of host plant, the next step is to characterize the mutations caused by that particular construct. If we target a diploid crop plant (e.g., rice), there are four potential outcomes: no mutation, a heterozygous mutation (only one allele is mutated), a biallelic mutation (both alleles are mutated, but the sequence of each allele is distinct), or a homozygous mutation (same mutation on both alleles) (Zischewski et al. 2016). The techniques employed to identify various kinds of mutations are mismatch cleavage assays [T7E1 assay, Surveyor™ nuclease assay (Transgenomic, Gaithersburg, MD, USA)], PCR-RE assay (Beumer et al. 2008), quantitative PCR high-resolution melting (qPCR-HRM) curve analysis technique (Yu et al. 2014) and sequencing.

5.4.1 T7E1 Assay

T7 endonuclease 1 (T7E1), an enzyme, identified from bacteriophage (Center and Richardson 1970), cleaves the cruciform DNA at 5′ end and provides a cost-effective and easy method for the detection of CRISPR/Cas-induced indel mutations. It recognizes structural abnormalities in dsDNA and cleaves the heteroduplexed DNA at specific sites (Dickie et al. 1987). It is the most commonly used methods for the detection of mutations created through CRISPR-Cas9-mediated genome editing. During genome editing through CRISPR technology, CRISPR/Cas constructs are introduced into plant cells using *Agrobacterium*-mediated or biolistic approaches resulting in the generation of targeted mutations. After few days, amplification of the genomic DNA surrounding the target region is carried out through PCR. The obtained amplicon is denatured and recomplexed by heating and subsequent slow cooling. During cleavage by Cas9 enzyme, if nonhomologous end joining occurs between the cut ends of DNA, the formation of heteroduplex occurs between PCR products of different lengths (e.g., mutant and WT amplicons), leading to DNA distortion that is recognized and cleaved by T7E1. The frequency of mutation is determined by the banding patterns of the cleaved products between control and experimental samples. However, the performance of the assay may be impacted by the length and identity of base pair mismatches, flanking sequence, secondary structure, and relative abundance of mutant sequences (Mashal et al. 1995; Vouillot et al. 2015). The sensitivity of T7E1 assay ranges between 0.5 and 5% (Kim et al. 2013). This method is suitable for detecting indels; however, SNPs cannot be identified by this method; rather it also tends to miss small indels in many cases (Qiu et al. 2004).

5.4.2 Surveyor™ Assays

Surveyor™ nuclease is an endonuclease derived from celery and is a site-specific enzyme. The sensitivity of surveyor assay is less (3%) as compared to T7E1 assay (Qiu et al. 2004) but is suitable for the detection of SNPs and small indels. The mechanism of action of surveyor assay relies on cutting both the DNA strands downstream of mismatch.

Therefore, the choice of mismatch cleavage using either T7E1 or Surveyor™ nuclease depends on which types of mutations are expected after genome editing. There are standard protocols which are provided with Surveyor™ nuclease assay kits. The mismatch cleavage assay usually underestimates the mutation frequency due to the preferential cleavage properties of each enzyme.

5.4.3 *Quantitative PCR High-Resolution Melting (qPCR-HRM) Curve Analysis*

During this method, amplification of DNA sequence surrounding genomic target is carried out through real-time PCR followed by incorporation of fluorescent dye and determining the melt curve analysis of amplicons (Dahlem et al. 2012). The principle behind HRMA method is during denaturation step of real-time PCR; intercalating dyes are released from dsDNA resulting in the loss of fluorescence. The data are collected over smaller temperature increments of 0.2 °C, followed by signal normalization and analysis as compared to the melt curves analyzed in typical quantitative PCR (qPCR) experiment. Melting temperature shifts and the shape of the melting curves can both provide useful information: homozygous allelic variants may cause a temperature shift in the melt curve compared to the wild-type homoduplex, whereas heteroduplexes representing heterozygous mutations change the shape of the melt curve due to the presence of mismatches (Taylor et al. 2010). HRMA is a sensitive and highly effective method as it does not involve direct handling of PCR sample and is a high-throughput screening method. It is a nondestructive method, i.e., it does not cause any harm to amplicons; therefore, they can further be analyzed by sequencing or heteroduplex mobility assay. This method is able to differentiate between homozygous wild type and homozygous mutants (Zischewski et al. 2016). The estimated detection limit in a ~100 bp amplicon is at least 2%, i.e., one mutant among 50 wild-type genomes for indels larger than 4 bp (Dahlem et al. 2012). Larger indels could not be detected by this method.

5.4.4 *Polymerase Chain Reaction-Restriction Enzyme (PCR-RE) Assay*

During CRISPR-Cas9-mediated genome editing, Cas9 nuclease cleaves double-stranded DNA three-base pair upstream of PAM sequence. If the site of any restriction endonuclease is present in the region upstream of PAM sequence, then the Cas9 nuclease will cut within a restriction enzyme site. The amplification of target locus and ~300–1000 bp of flanking material is achieved using 20–30-nucleotide-long fragments with Tm = 60 °C (Shan et al. 2014) followed by their digestion with appropriate restriction enzyme. The amplicons with no mutation (wild type) will be cleaved because no disruption of restriction site occurs, whereas the uncleaved bands will be amplicons of mutated region where there occurs loss of restriction enzyme site. The frequency of mutation can be observed from the uncleaved bands. PCR-RE assay can detect SNPs, small indels, and large indels (Zischewski et al. 2016). Subcloning of the uncleaved fragments followed by their sequencing can be used for detailed characterization of induced mutation.

5.4.5 Sequencing

CRISPR/Cas9-mediated mutations can be detected by amplification of the target sequence using specific primers followed by Sanger's sequencing and next-generation sequencing (NGS), which provides a direct proof for the type of mutation. This procedure could be laborious and time consuming. Analysis of large number of PCR amplicons sequenced through NGS can be carried through CRISPR-Genome Analyzer (CRISPR-GA) software (Güell et al. 2014). Large molecules can be identified through single-molecule real-time (SMRT) sequencing (Hendel et al. 2014), with an average read length of 8.5 kb.

5.5 Recent Advances in Genome Editing

Ever since the first report of the use of CRISPR/Cas9 to edit plant genome in 2013, there came many reports on the proof of concept, optimization of workflow in different plant species, advances in web-based tools to design sgRNA and identify off-target sites and incessant evolution of this technology in terms of identification/generation of SpCas9 variants to knock down a gene, identification of direct delivery agents, amenability of CRISPR/Cas system to interference (CRISPRi) or activation (CRISPRa), and use of choice-based editing enzymes to precisely edit a specific base in the gene sequence. Certainly, CRISPR/Cas9 has triggered a revolution in the laboratories which are using this technology for innovative applications in biology. Some of the recent advances of genome editing are briefly discussed below:

5.5.1 Cas9 Variants

5.5.1.1 *Cpf1*

Cpf1 is also an RNA-guided endonuclease of type II CRISPR system derived from *Prevotella* and *Francisella* bacteria. The *Cpf1* protein has a RuvC-like endonuclease domain that is similar to the RuvC domain of Cas9. Furthermore, *Cpf1* does not have a HNH endonuclease domain, and the N-terminus of Cpf1 does not have the alpha-helical recognition lobe of Cas9, and the size is smaller as compared to Cas9. It recognizes 5′-YTN-3′ as PAM sequence (where Y = pyrimidine and N = nucleobase) (Fonfara et al. 2016). Recently, CRISPR/Cpf1 system was used for targeting two genes, OsPDS and OsBEL, resulting in specific and heritable mutations in rice (Xu et al. 2016).

5.5.1.2 *Cas13*

Cas13 is sequence-specific RNA endonuclease. It is used for downregulating the transcripts of specific genes due to the presence of HEPN (higher eukaryotes and prokaryotes nucleotide binding) domain which is associated with RNase activity of single-stranded RNA. The most active orthologue of Cas13 enzyme is Cas13a isolated from *Leptotrichia wadei* which is being used in mammalian cells for knockdown of targeted RNA (Abudayyeh et al. 2017).

5.5.2 *DNA-Free Direct Genome Editing Using Ribonucleoproteins*

The expressed sgRNA and Cas9 can be made to enter into the plant cell either with the help of an agent (plasmid-carrying gene construct into the target cell by means of a bacteria like *A. tumefaciens* and *A. rhizogenes* or a virus like tobacco rattle virus and geminivirus) or by direct delivery of RNA (in the form of in vitro transcripts (IVTs), Cas9 mRNA) and protein via protoplast transfection or microprojectile bombardment or in the form of a pre-assembled ribonucleoprotein complex. *Agrobacterium*-mediated genetic transformation, PEG-mediated protoplast transformation, and particle bombardment have been extensively used as conventional delivery methods for CRISPR/Cas9 reagents (Jiang et al. 2013; Shan et al. 2013, 2014). However, its wider applicability is limited by off-target effects, unwanted and random integration of DNA segments from plasmid vectors into the host genome (Kim et al. 2014; Koo et al. 2015).

Unlike the plasmid-based methods, ribonucleoprotein (RNP) complex has come up as a very efficient method for the direct delivery of active components of CRISPR machinery into a plant cell. These RGEN components may be prepared separately and assembled in vitro and subsequently introduced into the plant genome (classical chemical transfection) for the desired genetic modifications. In vector delivery method, there is a possibility of integration of remnants of the constructs into the plant genome, but RNP completely rules out any such possibility. RNPs act immediately upon delivery with highest mutation frequency 1 day after delivery into the cell. sgRNA and Cas9 protein have a decreased functional time within the cell as they rapidly get degraded in vivo (by the action of endogenous proteases) after editing and, therefore, leaving no trace of foreign DNA in the plant genome. Accordingly, the chances of cleavage at off-target sites are greatly reduced (Kim et al. 2014). Woo et al. (2015) first demonstrated the use of this next-generation plasmid-independent CRISPR/Cas9 genome editing approach in plants by transfecting the protoplasts of *Arabidopsis*, tobacco, lettuce, and rice and obtaining targeted mutagenesis up to 46% in regenerated protoplasts. Liang et al. (2017) succeeded in editing two genes *TaGW2* and *TaGASR7* by delivering CRISPR/Cas9 RNPs via particle bombardment. The application of this direct delivery mode has also been tested in other food

crops like maize (Svitashev et al. 2016) and soybean (Kim et al. 2017) and horticultural crops like apple and grapes for increasing resistance to powdery mildew and fire blight disease (Malnoy et al. 2016). To adopt this next-generation, RGEN RNP tool to develop vector-free plants, different options may be exploited depending upon the amenability of different species to these methods. These methods include PEG-mediated protoplast transformation using microinjection mesoporous silica nanoparticles (MSNs) and cell-penetrating peptides (Martin-Ortigosa and Wang 2014; Jensen et al. 2014; Masani et al. 2014; Woo et al. 2015).

RNP has a much broader spectrum of applications to offer than mere gene disruption. It dramatically reduces off-target mutations without compromising on-target efficiency. It offers high specificity, paving way for precision crop breeding and developing DNA-free genetically edited crop plants (Kanchiswamy 2016). Though the frequency of mutagenesis obtained via RNP delivery or use of RNA is low, chances of off-target mutagenesis are considerably reduced. RNP delivery also enables HR-mediated precise gene editing as seen in the case of endogenous *ALS2* where a 127-base single-stranded repair template for HR was co-delivered (Maize, Mark Cigan group). Truly, RNP has marked the evolution of next generation of GE tools. As mutations induced by these protein complexes do not fall under the current regulatory legislations, they may be qualified as non-GMOs, promoted for commercialization, public acceptance, and practical cereal improvement (Wolter and Puchta 2017).

5.5.3 CRISPR-Cas9-Mediated Base Editing

Homology-directed repair (HDR) or targeting-induced local lesions in genome (TILLING) has been used for a long time for generating point mutations in crops. Genome editing in plants via NHEJ or HDR pathway involves double-strand break repair. HDR has been found to be relatively inefficient due to the competition by NHEJ, the dominant pathway to repair DSB in plants. Moreover, the delivery of DNA repair template into the cell is also quite challenging.

Targeted base editing offers itself as a very promising technique that may enable precise and efficient base replacement in the target locus (Lu and Zhu 2017) without the need for a foreign DNA donor or dsDNA cleavage. It may offer a direct and irreversible conversion of one target base into another in a programmable manner (Komor et al. 2016). Since this approach does not involve a donor DNA template, it avoids the chances of random integration of donor DNA in the genome of the edited plants. The first generation of experimental optimization has been conducted for converting blue fluorescent protein (BFP) into a green fluorescent protein (GFP) by fusing rat cytidine deaminase APOBEC1 with a Cas9 variant which may be a nickase or a dead Cas9. CRISPR-Cas9 nickase-cytidine deaminase fusion protein enabled editing of a single base in the plant genome when directed by sgRNA. Zong et al. (2017) used CRISPR-Cas9 nickase-cytidine deaminase fusion protein to achieve targeted conversion of cytosine to uracil/thymine from position P3 to P9

within the protospacer region of *OsCDC48*, *OsNRT1.1B*, and *OsSPL14* in rice, *TaLox2* in wheat, and *ZmCENH3* in maize with a mutagenesis frequency up to 43.48%. Relative to gene disruption or replacement strategy, indel frequency was found to be greatly reduced. Accordingly, targeted nucleotide substitution within the desired locus may be made without the need for double-strand break (NHEJ) or the introduction of any foreign DNA (HDR). Lu and Zhu (2017) too tested the applicability of this strategy in rice for two agronomically important genes—*NRT1.1B* (nitrogen transporter) and *SLR1* (DELLA proteins).

5.6 Applications of Genome Editing for Crop Improvement

CRISPR/Cas9 RGEN system has completely revolutionized the genome editing platform. It may be used for sequence-specific integration of more than one gene for pyramiding useful traits in elite cultivars that may help develop improved varieties in a shorter time. It may be utilized for the creation and use of novel allelic variants, a new source of genetic variation, and broaden the spectrum of existing genetic variability for breeding crops. Hybridizing the transgenic plant carrying CRISPR-Cas9 system with elite cultivar may provide non-transgenic genome modification in plants which may address ethical concerns raised against commercialization of transgenic plants. Further, the introduction of desired genetic/epigenetic variation via CRISPR-Cas9 system may provide a promising platform to engineer gene networks in their native context to improve quantitative traits of agronomic importance like increased yield, enhanced tolerance to diseases, insect pests, and abiotic stress. Successful genome editing has been carried out in different crops like wheat, rice, maize, sorghum, barley, and *Brassica* (Table 5.1). Plant diseases caused by microorganisms are the major factor that reduces quality and yield of crops. Powdery mildew is one of the most common plant diseases caused by fungal species. Wang et al. (2014) were successful in inducing selective mutation in one of the three homeoalleles of mildew resistance locus (*MLO*) in wheat with mutation frequency of 5.9%. Resistance to powdery mildew through CRISPR/Cas technology was also achieved by Zhang et al. (2017) by knocking out of *TaEDR1* gene. The quality of rice was improved by knocking out the expression of starch-branching enzymes (*OsSBEI* and *OsSBEII)*, genes for reducing the content of amylopectin and increasing amylose content in rice (Sun et al. 2017). Table 5.1 gives a comprehensive elaboration of the use of CRISPR-Cas strategy for improving important traits in different crop species.

Table 5.1 Applications of genome editing in various field crops

Sr. no.	Crop	Target gene	Trait	Delivery method	Knockout/HR	Remarks	Reference
1	Wheat	DQ2.5-glia-α (celiac disease epitopes)	Gluten allergenicity	*Agrobacterium* mediated	Knockout	Transgene-free lines identified	Sánchez-León et al. (2018)
2		Fluorescent protein genes—GFP, BFP, dsRED	Selection of transformation events	Wheat dwarf virus-based replicon system using biolistic transformation	Knockout, HR also attempted	Gene targeting occurred in all three homeoalleles (A, B, and D)	Gil-Humanes et al. (2017)
3		*TaEDR1* (*enhanced disease resistance 1 locus*)	Resistance to powdery mildew	*Agrobacterium* mediated	Knockout	Triple knockout mutant with frameshift	Zhang et al. (2017)
4		*TaGW2*	Grain weight	Direct delivery of ribonucleoprotein complexes through particle bombardment	Knockout	Transgene-free lines, no foreign DNA used, mutation frequency of 0.19%	Liang et al. (2017)
5		*TaGASR7*	Grain length and grain width				
6		*TaGASR7*	Grain length and grain width	Particle bombardment	Knockout	TECCDNA and TECCRNA approach developed for transient expression of sgRNA, Cas9, and in vitro transcripts, respectively, selection-free medium used, mutation frequency of 9.5% with TECCDNA and 1.1% with TECCRNA	Zhang et al. (2016)
7		*TaDEP1*	Inflorescence architecture				
8		*TaNAC2*	Regulator of shoot branching				
9		TaPIN1	Auxin-dependent adventitious root emergence				

(continued)

Table 5.1 (continued)

Sr. no.	Crop	Target gene	Trait	Delivery method	Knockout/HR	Remarks	Reference
10		*Q locus*, *TaGW2*, *TaLpx-1*	Agronomic traits associated with shattering habit, grain weight	Protoplast transformation	Knockout	Multiplex gene editing using construct with several gRNA-tRNA units under the control of a single promoter	Wang et al. (2016c)
11		TaMLO-A1 (powdery mildew resistance locus)	Disease resistance	*Agrobacterium* mediated	Knockout	Powdery mildew-resistant plants with 5.6% mutation frequency	
12		Inox (inositol oxygenase)	Biogenesis of plant cell wall	*Agrobacterium* mediated	Knockout	18–20% mutation frequency, formation of chimeric plants	Upadhyay et al. (2013)
13		PDS (phytoene desaturase)	Biosynthesis of chlorophyll			18–20% mutation frequency, albino phenotype	
14	Rice	eIF4G (translation initiation factor 4 gamma gene)	Resistance to RTSV (rice tungro spherical virus)	*Agrobacterium* mediated	Knockout	Mutation rates ranged from 36.0% to 86.6% (frameshift mutations resulted in providing resistance), which were further transmitted to next-generation, no off-targets	Macovei et al. (2018)
15		*NRT1.1B*	Nitrate transporter gene	*Agrobacterium* mediated	Homology-directed repair	Replacement of *NRT1.1B* gene (encoding for nitrate transporter) of japonica rice with the corresponding elite allele in Indica rice for improved nitrogen use efficiency	Li et al. (2018)

16		*OsSBEI*	High-amylose rice	*Agrobacterium* mediated	Knockout	From 40 *SBEI* mutant lines, 12.5%, 27.5%, and 40% were heterozygous, biallelic, and homozygous *SBEI* mutant lines	Sun et al. (2017)
17		*OsSBEII*				From 30 *SBEIIb* mutant plants, 6.7%, 36.6%, and 26.7% were heterozygous, biallelic, and homozygous plants	
18		*OsNramp5*	Metal transporter gene	*Agrobacterium* mediated	Knockout	Development of indica rice lines with low Cd accumulation	Tang et al. (2017)
19		*OsERF922*	Ethylene-responsive factor (negative regulator of blast resistance)	*Agrobacterium* mediated	Knockout	42% genome editing efficiency (21 *ERF922* mutant plants were obtained from 50 T_0 transgenic plants) for providing resistance against *Magnaporthe oryzae* (blast resistance)	Wang et al. (2016a)
20		*OsAOX1a*	Alternate oxidase genes (involved in mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis)	*Agrobacterium* mediated	Knockout	Stable biallelic T_0 transgenic rice plants were obtained by introduction of CRISPR/Cas9 construct into cv. Nipponbare	Xu et al. (2015)
21		*OsAOX1b*					
22		*OsAOX1c*					

(continued)

Table 5.1 (continued)

Sr. no.	Crop	Target gene	Trait	Delivery method	Knockout/HR	Remarks	Reference
23		*OsPDS*	Chlorophyll synthesis	*Agrobacterium* mediated	Knockout	CRISPR-Cas9 system was used for editing of different genes of rice varieties Nipponbare (*Oryza sativa* L. ssp. japonica) and Kasalth (*Oryza sativa* L. ssp. indica). 44.4% of T_0 plants carried mutation; no off-target effects were observed	Zhang et al. (2014)
24		*OsPMS3*	Photoperiod-sensitive male sterility				
25		*OsEPSPS*	Herbicide resistance				
26		*OsDERF1*	Hydrolysis of proteins				
27		*OsMYB1 and OsMYB5*	Transcription factors binding to promoters of storage protein glutelin				
28		*OsROC5*	Rice outmost cell-specific gene				
29		*OsSSP*	Encodes signal peptide peptidases in shoot apex				
30		*OsSWEET11*	Bacterial blight susceptibility genes	*Agrobacterium* mediated	Knockout	44.4% of T_0 plants carried mutation. No off-target effects were observed	Zhang et al. (2014)
31		*OsSWEET14*					
32		*COA1* (chlorophyll A oxygenase)	Chlorophyll synthesis	*Agrobacterium* mediated	Knockout	Silencing of CAO1 gene leads to pale green phenotype	Miao et al. (2013)
33		LAZY1	Encodes chromodomain protein required for chloroplast signal recognition pathway			Silencing of *LAZY1* gene leads to tiller-spreading phenotype	

34		*BEL*	Herbicide resistance gene	*Agrobacterium* mediated	Knockout	Biallelic mutant plants were sensitive to bentazon with mutation frequency ranging from ~2% to ~16%	Xu et al. (2014)
35	Maize	*zb7*	Chloroplast development	*Agrobacterium* mediated	Knockout	*dmc1* promoter-controlled CRISPR/Cas9-based genome editing into callus derived from immature embryos for specific expression in meiocytes. Homozygous or biallelic mutants were observed in 66% of T_0 plants which were further passed onto the T_1 generation	Feng et al. (2018)
36		*zyp1*	Controlling central protein element of synaptonemal complex				
37		*smc3*	Structural maintenance of chromosome				
38		*ARGOS8*	Negative regulator of ethylene responses	Particle bombardment	Knockout	Plants having mutated *ARGOS8* locus showed improved grain yields of 5 bushels per acre under drought conditions	Shi et al. (2017)
39		*Argonaute 18* (*ZmAgo18a* and *ZmAgo18b*)	Play primary role in miRNA and siRNA pathways	*Agrobacterium* mediated	Knockout	Targeted mutagenesis was observed in 60% of putative transgenic callus lines which was observed in T_0 plants and was further transmitted to T_1 generation	Char et al. (2017)
40		*Dihydroflavonol 4-reductase*	Involved in flavonoid synthesis				

(continued)

Table 5.1 (continued)

Sr. no.	Crop	Target gene	Trait	Delivery method	Knockout/HR	Remarks	Reference
41		*PSYI (phytoene synthase gene)*	Encoding for carotenoid synthesis	*Agrobacterium* mediated	Knockout	Out of T_0 putative lines (agro-infected immature embryos), two showed white leaf stripe phenotype, and one showed albino phenotype (seedling stage)	Xu et al. (2016)
42		*LIG1 (liguleless 1)*	Plays role in leaf development	Direct delivery of pre-assembled ribonucleoprotein complexes through particle bombardment	Knockout	RNP complexes were introduced targeting the three genes, and mutation frequency was 2.7% to 9.7%: *ALS2* mutant becomes resistant to chlorsulfuron; MS26 and MS45 mutants became male sterile	Svitashev et al. (2016)
43		*ALS2 (acetolactate synthase)*	Catalyzes first step in synthesis of amino acids		Homologous recombination to introduce a single amino acid change (proline to serine)		
44		*MS26 and MS45 (two male fertility genes)*	Providing male fertility		Knockout		
45		*ZmIPK* (*inositol phosphate kinase gene)*	Production of phytic acid	*Agrobacterium* mediated	Knockout	gRNA vector driven by U3 promoter resulted in transformation frequencies ranging from 16.4% to 19.1%	Liang et al. (2014)
46	Sorghum	dsRED2 (red fluorescent protein)	Selection of transformation events	*Agrobacterium* mediated	Knockout	Uniform GFP and dsRED expression (28% mutation frequency)	Jiang et al. (2013)
47	Barley	ENGase (endo-N-acetyl-β-D-glucosaminidase)	Modification of N-glycans in cereals	*Agrobacterium* mediated	Knockout	No macroscopic changes in phenotype (78% mutation frequency)	Kapusi et al. (2017)
48	*Brassica*	ALCATRAZ	Shattering from mature grain	*Agrobacterium* mediated	Knockout	Increased shattering resistance	Braatz et al. (2017)

5.7 Regulatory Considerations

The next wave of genetically edited crops will soon make its way from laboratory into the market. But before that, these products should be able to stand the test of field trials, proceed through multiple regulatory loops, and win over the concerns of the masses assuring general public acceptance. USDA and APHIS designate a crop as genetically modified (GM) if it is produced using a plant pathogen or if it contains a foreign gene. These GMOs are subjected to stringent regulatory procedures before commercialization. The modified plants generated by CRISPR-Cas9 system are by and large indistinguishable from naturally occurring mutant crops or that developed by conventional breeding approaches. Regulatory authorities around the world are currently considering how to handle the plants that lack a transgene yet carry targeted mutations such as those created by NHEJ (Kuzma and Kokotovich 2011). USDA does not regulate plants if they contain targeted mutagenesis occurring by self-repair mechanism. GE canola; non-browning, acrylamide-safe potatoes; and GE mushrooms have already been approved by the USDA and waived off from GMO regulations (Waltz 2016; Haun et al. 2014).

One way to escape GMO legislation and produce non-transgenic genome-edited plants is to switch from DNA to RNA or proteins, particularly the use of ribonucleoprotein complexes (Wolter and Puchta 2017). In addition to these GMO-based legislative regulations, the unresolved patent claims are delaying the widespread use of CRISPR/Cas9 technology. The rapid resolution of license negotiation and the establishment of licensing free structure shall give a tremendous boost to this breakthrough technology to help in solving the problem of food shortage and environmental protection.

5.8 Conclusion

CRISPR/Cas is a versatile, precise, and efficient tool that can be used to target any region of the genome of an organism by changing the gRNA sequence. It will help in annotating large amounts of sequence information which will further help in discovering the genomes of different crop plants by overcoming the limitations of searching plant populations with adequate variation and conventional crossing methods. The simple knockout strategy holds significant promise for creating targeted allelic variation for crop improvement. Base editing and homology-dependent insertion of new DNA further expand these possibilities. Ribonucleoprotein-based methodologies are likely to reduce the possibilities of apprehended off-target genome modifications. The CRISPR/Cas system thus needs to be integrated in the crop improvement programs as a quicker, safer, and accurate methodology to take advantage of the presently available vast genome sequence data to develop crop varieties for the future needs of the farmers and consumers.

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Chapter 6
Utilization of Wild Species for Wheat Improvement Using Genomic Approaches

Satinder Kaur, Suruchi Jindal, Maninder Kaur, and Parveen Chhuneja

Abstract Wheat is one of the most important food crops in the world in terms of the area, production, and nutrition. It can grow in varied tropical and temperate climates ranging from a few meters to more than 3800 m above sea level. In spite of a wide range of climatic adaptability, a number of biotic and abiotic stresses limit its yield stability. Wild wheat relatively belonging to primary, secondary, and tertiary gene pool contains untapped variation for wheat improvement, both for biotic and abiotic stresses that could be incorporated in cultivated wheat. Diversity of primary gene pool can be incorporated by simple hybridization methods, while diversity of secondary gene pool of *Triticum* and *Aegilops* species, sharing one genome common with wheat, can be transferred with slight manipulations in hybridization. Species belonging to the tertiary gene pool are more distantly related to wheat. To transfer variation from these species to common wheat, special cytogenetic manipulations are required. Availability of various genomic resources of wild species of wheat is making an easy way to reach the genes which become possible because of recent developments in sequencing technologies. These platforms enabled the sequencing of progenitor species of wheat like *T. urartu*, *Aegilops tauschii*, wild emmer wheat, and *Triticum monococcum* as well as transcriptome sequencing of non-progenitor species like *Aegilops sharonensis* and *Agropyron cristatum*. Sequence data thus obtained from wild species of wheat hold the potential for the improvement of wheat crop. The genic sequences or expressed sequence tags (ESTs) obtained from the wild species are used to design SNP chips of data capacity 35 K and 820 K which are being used to map and fine map agronomically important genes. Also the technique of flow cytometry enabled the flow sorting of larger genomes which allows the focus only on specific chromosomes containing genes of interest. Besides latest innovation in RenSeq and MutRenSeq allowed the mapping and cloning of disease resistance genes more fast and reliable.

Keywords Wheat · Wild germplasm · Progenitor · Non-progenitor · *Aegilops* · Genome · Transcriptome · Resistant gene · Biotic stress · Abiotic stress

S. Kaur (✉) · S. Jindal · M. Kaur · P. Chhuneja
School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, India
e-mail: satinder.biotech@pau.edu; suruchi-coasab@pau.edu; pchhuneja@pau.edu

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6.1 Introduction

Wheat is the most important winter cereal crop in India and serves as the staple food for more than one billion population. It contributes approximately 14% to the world wheat basket and holds the global share of 11% of area under cultivation of wheat.

6.1.1 Brief History: India

Historical evidence of wheat cultivation comes from the remnant of wheat grains *Triticum aestivum* subspecies *sphaerococcum* popularly known as Indian wheat during Indus Valley Civilization about 5000 years back. During the 1950s wheat requirement of India was mainly fulfilled by import from other countries. Yield level of local varieties was poor due to low-yielding nature of tall Indian wheat which is prone to lodging under high-fertility conditions, and wheat production was merely 6.46 million tons. With introduction of dwarf spring wheat strains, possessing the Norin-10 dwarfing genes and several measures to stimulate food and wheat production went up to 12 million tons in 1964. Hybridization between Mexican strains and Indian varieties resulted in many high-yielding and rust-resistant strains in different parts of the country. With further efforts wheat production rose to nearly 17 million tons in 1968. This break in yield stagnation was described as "Green Revolution." Wheat crop has exhibited a robust growth trend since the onset of the Green Revolution in 1968. In 2001 our wheat production was 74 million tons, as compared to 6 million tons during the1950s. Much of the increase in wheat production has come from productivity improvement. Varietal diversification and pyramiding of genes for tolerance to biotic and abiotic stresses had great role in increased productivity. Now wheat is grown in India in an area of about 30 million ha with a production of 93 million tons. The normal national productivity is about 2.98 tons/ha. The major wheat-producing states are Uttar Pradesh, Punjab, Haryana, Madhya Pradesh, Rajasthan, Bihar, Maharashtra, Gujarat, Karnataka, West Bengal, Uttarakhand, Himachal Pradesh, and Jammu and Kashmir. Uttar Pradesh stands first followed by Madhya Pradesh and Punjab. In productivity, Punjab stands first followed by Haryana and Uttar Pradesh.

6.1.2 Brief History: The World

The total area under wheat in the world is around 225.62 million hectares with a production of 685.6 million tons (2009–2010). The normal world productivity is 3039 Kg/ha. The major wheat-producing countries are China, India, the United States, France, Russia, Canada, Australia, Pakistan, Turkey, the United Kingdom, Argentina, Iran, and Italy. These countries contribute about 76% of the total world

wheat production. India stands first in area and second in production next to China in the world. India's share in world wheat area is about 12.40%, whereas it occupies 11.77 % of share in the total world wheat production.

In the race of improving production, we somehow losses the diversity of the crop leading to few varieties with similar gene pools. This gives open invitation to various biotic and abiotic stresses that too on a massive scale. Thus in the present scenario, there is a need to exploit the existing wild progenitor and non-progenitor resources to harness the diversity. There is hardly any scope for expansion of area under wheat. The main emphasis would be on increasing the productivity of wheat by improving the biotic and abiotic stresses and introducing diversity.

6.2 Phylogeny and Origin of Polyploid Wheats

Wheat (*Triticum* spp. L.) belongs to the family Poaceae, a family of major crop plants providing staple food, rice (*Oryza sativa* L.), maize (*Zea mays* L.), barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), and oat (*Avena sativa* L.). It belongs to the tribe *Triticeae* along with oat and barley and contains more than 15 genera and 300 species. Wheat was first classified by Linnaeus in 1753, and further research separated wheat into three groups, viz., diploid ($2n = 14$), tetraploid ($2n = 28$), and hexaploid ($2n = 42$) chromosomes. There are six biological species of wheat at three ploidy levels: diploid level, *Triticum monococcum* (A^mA^m) and *T. urartu* (AA); tetraploid level, *T. turgidum* (BBAA) and *T. timopheevii* (GGAA); and hexaploid level, *T. aestivum* (BBAADD) and *T. zhukovskyi* ($GGAAA^mA^m$). Of these species, *T. urartu* exists only in its wild form, whereas *T. monococcum*, *T. turgidum*, and *T. timopheevii* have both a wild and a domesticated form. *T. aestivum* and *T. zhukovskyi* exist only as cultivated forms. Wheat lineage corresponds to *T. urartu*, *T. turgidum*, and *T. aestivum*, while *T. monococcum*, *T. timopheevii*, and *T. zhukovskyi* form a separate lineage different to the evolution of the principal wheat. Hybridization of *T. urartu* with a close related species of *Aegilops speltoides* (SS) give rise to *Triticum turgidum* (Sarkar and Stebbins 1956). The domestication of the resulting wild emmer (*T. turgidum* ssp. *dicoccoides*) gave rise to domesticated emmer (*T. turgidum* ssp. *dicoccon* genomes BBAA), and finally free-threshing tetraploid wheat, *T. turgidum* ssp. *durum*, has been evolved. Sax (1922) based on the cytological data on chromosome pairing in interspecific hybrids among species of different ploidy levels indicated that *T. monococcum* and *T. turgidum* have one genome in common, while *T. turgidum* and *T. aestivum* share two genomes in common. Kihara (1944) unequivocally demonstrated that *Ae. tauschii* was the D-genome donor of bread wheat which arose from a hybridization between *T. turgidum* and *Ae. tauschii* var. *strangulata* about 7000 years ago (Dvorak et al. 1998).

6.3 Gene Pools of Wheat

On the basis of their genomic constitution, the wild relatives of wheat can be classified into primary, secondary, and tertiary gene pool (Friebe et al. 1996). Species belonging to the primary gene pool share homologous genomes with cultivated wheat. This group includes land races of *T. aestivum*, the wild (*T. dicoccoides*) and cultivated forms of *T. turgidum*, and donor species of the A and D genomes of bread wheat, *T. monococcum*, *T. urartu*, *T. boeoticum*, and *Ae. tauschii*. These species are easily intercrossable (Friebe et al. 1996). Some cross combinations require embryo rescue, but no cytogenetic manipulation procedures are necessary. Diploid A-genome species, donor of A genome of tetraploid and hexaploid wheat, includes two species of einkorn wheat: *Triticum monococcum* Linn (genome A^mA^m) and *Triticum urartu* Thum ex Gand (genome A^u A^u) (Gulbitti-Onarici et al. 2007). The genome size and genome content of both the genomes are the same (Ozkan et al. 2010), but their hybrid is sterile (Johnson and Dhaliwal 1976). *T. monococcum* comprises of wild and cultivated subspecies of the wild *T. monococcum* subsp. *boeoticum* Boiss. (Gulbitti-Onarici et al. 2009) and its domesticated form *T. monococcum* subsp. *monococcum* (Sourdille et al. 2001); *T. boeoticum* are widespread in Western Asia and southern Balkans (Harlan and Zohary 1966) and are extensive in rocky areas of southeastern Turkey (Harlan and Zohary 1966) . Cultivated species *T. monococcum* L. ssp. *monococcum* L. ($2n = 14$) originated in the Karacadağ mountains of Turkey (Heun et al. 1997) and was widely cultivated during the Neolithic period. The hybrids of these two species *T. monococcum* subsp. *boeoticum* and *T. monococcum* subsp. *monococcum* are fertile. *Triticum urartu* is A-genome donor of *T. durum* or macaroni wheat *T. turgidum* (AABB), *T. timopheevii* (AAGG), and common wheat *T. aestivum* (AABBDD). In contrast, *T. monococcum* has only been used for the generation of *T. zhukovskyi* (*AmAmAAGG*) (Dubcovsky et al. 1995). *Aegilops tauschii* Coss., a diploid self-pollinating goat grass species, has contributed the D genome to common wheat. Hybridization of *Ae. tauschii* (DD) with tetraploid wheat, *T. turgidum* L. (AABB), about 7000 years ago led to the development of hexaploid wheat *Triticum spelta* (L) Thell (AABBDD) from which common wheat evolved.

The tetraploid wheat group is composed of ten species, carrying either AB or AG genome and growing in a wider range of environments than diploids. Cultivated emmer (*T. dicoccum*) was the predominant cultivated wheat during the Neolithic age. During the Bronze Age, the naked tetraploid wheats slowly displaced emmer wheat which however remains an important crop in Ethiopia and Yemen. *T. dicoccoides* has been recognized as a valuable source of powdery mildew and leaf rust resistance.

The secondary gene pool of common wheat includes the *Triticum* and *Aegilops* species that have at least one genome in common or partially common with wheat including *T. timopheevi* (AAGG), *T. zhukovskyi* (GGAAAA), and *Ae. speltoides* (SS). These species can be intercrossed, but only recombination between homologous chromosomes is possible. It is also possible to transfer gene from

nonhomologous genome but with special cytogenetic manipulations. The genus *Aegilops* belongs to the tribe *Triticeae*, subtribe *Triticinae*. The genus *Aegilops* consists of 22 species of which 10 are diploid, 10 are tetraploid, and 2 are hexaploid with basic chromosome number $x = n = 7$ and six different genomes as C, D, M, N, S, and U (Van Slageren 1994). The species have been classified into *Aegilops* (8 species), *Comopyrum* (2 species), *Cylindropyrum* (2 species), *Sitopsis* (5 species), and *Vertebrata* (5 species). *Aegilops* represents the largest part of the secondary gene pool of wheat, and several species have been used in crop improvement programs (Kilian et al. 2011). Some *Aegilops* species (*Ae. speltoides* and *Ae tauschii*) participated in wheat evolution and played a major role in wheat domestication. These species represent a rich source of genes that can be utilized in wheat improvement. For example, *Ae. umbellulata* (UU), *Ae. peregrina* (UUSS), *Ae. caudata* (CC), and *Ae. comosa* (MM) are known sources of important agronomic traits such as tolerance to biotic (BYDV, cereal cyst nematode, Hessian fly, leaf rust, stripe rust, tan spot, and powdery mildew) and abiotic stresses (drought, frost, heat, salt, Zn deficiency) and nutritional and bread-making quality.

Species belonging to the tertiary gene pool are diploid and polyploidy species more distantly related to wheat. Their chromosomes are not homologous to those of wheat. About 325 species are known in the tribe *Triticea*e, of which 250 are perennials (Dewey 1984), and the rest are annuals including *Secale cereale* (RR), *Thinopyrum elongatum* (EE), *Elymus* (SSHHYY), and *Thinopyrum intermedium* (JJEESS) species. These species are completely reproductively isolated from wheat because their genomes are nonhomologous to those of wheat and special cytogenetic techniques are required to ensure compensating transfer with least linkage drag for commercial exploitation of introgressed derivatives. Though gene transfer from distant relatives is more difficult, it has been established that the more distant from wheat the relative is, the more likely it is to have new unique genes to enrich gene pool of wheat. Species such as *Agropyron elongatum* (Host) Beauv. and *Agropyron cristatum* Gaertn. are reported to contribute to higher grain yields in wheat growing under optimal conditions. In certain wheat backgrounds, chromosome 7 Ag from *A. elongatum* increases grain yield up to 8% and carries leaf (Lr) and stem rust (Sr) resistance genes *Lr19* and *Sr25*, respectively. One of the most widely used tertiary gene pools is rye (*Secale cereale* L.), which is well-documented as a rich source of biotic and abiotic resistance/tolerance. During the 1990s, the 1BL.1RS translocation was present in 60% of wheat descending from lines developed at the International Maize and Wheat Improvement Center (CIMMYT) and nearly half of the commercial varieties.

6.4 Biotic Stress Resistance from Wild Germplasm of Wheat

Biotic stresses are major quencher of yield in wheat crop. A large number of genes have been transferred from wild species into cultivated wheat to meet the demand of this staple food worldwide (Tables 6.1 and 6.2).

Table 6.1 List of biotic and abiotic stress resistance genes, transferred from wild progenitor species

Gene	Source	Chromosome	Reference
Leaf rust resistance genes			
Lr63	*T. monococcum*	3AS	Kolmer et al. (2010)
Lr21	*Ae. tauschii*	1DS	Rowland and Kerber (1974) Huang and Gill (2001)
Lr22a		2DS	Hiebert et al. (2007)
Lr32		3D	Thomas et al. (2010)
Lr39/Lr41		2DS	Raupp et al. (2001) Singh et al. (2004)
Lr40		1DS	Spielmeyer et al. (2000)
Lr42		1DS	Liu et al. (2013)
Lr43		7DS	Hussien et al. (1997)
Lr28	*Ae. speltoides*	4AL	Cherukuri et al. (2005)
Lr35		2B	McIntosh et al. (1984)
Lr36		6BS	Gold et al. (1999)
Lr47		7AS	Helguera et al. (2003)
Lr51		1BL	Helguera et al. (2005)
Lr66		3A	Marais et al. (2009)
Lr53	*T. dicoccoides*	6BS	Dadkhodaie et al. (2010)
Lr64		6AL	Kolmer et al. (2008)
Stripe rust resistance genes			
Yr28	*Ae. tauschii*	4DS	Singh et al. (2000)
Yr15	*T. dicoccoides*	6BS	Sun et al. (1997)
Yr35		6BS	Dadkhodaie et al. (2011)
Yr36	*T. dicoccoides*	6BS	Uauy et al. (2005)
Stem rust resistance genes			
Sr21	*T. monococcum*	2AL	Chen et al. (2015)
Sr22		7AL	Olson et al. (2010)
Sr35		3AL	Saintenac et al. (2013)
Sr33	*Ae. tauschii*	1DL	Periyannan et al. (2013)
Sr45		1DS	Periyannan et al. (2014)
Sr46		2DS	Singh et al. (2011)
Sr32	*Ae. speltoides*	2AL, 2BL	Mago et al. (2013)
Sr39		2B	Gold et al. (2002) Mago et al. (2009)
Sr47		2BL	Faris et al. (2008)
Powdery mildew resistance genes			
Pm1b	*T. monococcum*	*7AL*	Hsam et al. (1998)
Pm1c		*7AL*	Sears and Briggle (1969) Hartl et al. (1995)
Pm4d		*2AL*	Schmolk et al. (2012)
Pm4a		*2AL*	

(continued)

Table 6.1 (continued)

Gene	Source	Chromosome	Reference
Pm25		*1A*	Shi et al. (1998)
Pm2	*Ae. tauschii*	*5DS*	Qiu et al. (2006)
Pm19		*7D*	Lutz et al. (1995)
Pm34		*5DL*	Miranda et al. (2006)
Pm35		*5DL*	Miranda et al. (2007)
Pm58		*2DS*	Wiersma et al. (2017)
Pm1d	*T. spelta* var. *duhamelianum*	*7AL*	Hsam et al. (1998)
Pm12	*Ae. speltoides*	*6BS*	Jia et al. (1996)
Pm32		*1BL*	Hsam et al. (2003)
Pm53		*5BL*	Petersen et al. (2015)
Pm3k	*T. dicoccoides*	*1AS*	Yahiaoui et al. (2009)
Pm16		*4A*	Chen et al. (2005)
Pm26	*T. turgidum* var. *dicoccoides*	*2BS*	Rong et al. (2000)
Pm30		*5BS*	Liu et al. (2002)
Pm31		*6AL*	Xie et al. (2004)
Pm36		*5BL*	Blanco et al. (2008)
Pm41		*3BL*	Li et al. (2009)
Pm42		*2BS*	Hua et al. (2009)
Fusarium head blight resistance genes			
QTL	*T. dicoccum*	*4B, 6A, 6B, 7A*	Buerstmayr et al. 2012
Qfhs.ndsu-3AS	*T. dicoccoides*	*3AS*	Otto et al. (2002)
Qfhs.fcu-7AL	*T. dicoccoides*	*3AS*	Kumar et al. (2007)
Wheat blast resistance genes			
Rmg	*T. dicoccoides*		Tagle et al. (2015)
Cold tolerance genes			
FrA1	*T. monococcum*	*5AL*	Va´gu´jfalvi et al. (2003)
FrA2	*T. monococcum*	*5AL*	Va´gu´jfalvi et al. (2003)
TmCBF5	*Triticum monococcum* L.	7A	Miller et al. (2006)
TmCBF18	*Triticum monococcum* L.	6A	Miller et al. (2006)
Salt tolerance genes			
TmHKT1;5-A	*T. monococcum*	–	Munns et al. (2012)
Drought tolerance genes			
TdicDRF1	*T. turgidum* ssp. *dico*		Lucas et al. (2011a)
TdTMKP1	*T. turgidum* L. ssp. *durum*		Zaidi et al. (2010)
TdP1P2;1, *TdP1P2;2*	*T. turgidum* L. ssp. *durum*		Ayadi et al. (2011)
TdicATG8	*T. dicoccoides*		Kuzuoglu-Ozturk et al. (2012)
TdicTMPIT1	*T. dicoccoides*		Lucas et al. (2011)

(continued)

Table 6.2 List of biotic and abiotic stress resistance genes, transferred from wild non-progenitor species and tagged with molecular markers

Gene	Source	Chromosome	Reference
Leaf rust resistance genes			
Lr61	*T. turgidum*	6BS	Herrera-Fossel et al. (2008)
Lr9	*Aegilops umbellulata*	6BL	Schachermayr et al. (1994)
Lr76	*Ae. umbellulata*	5DS	Bansal et al. (2016)
Lr18	*T. timopheevii*	5BL	Yamamori (1994)
Lr50	*T. timopheevii* subsp. *armeniacum*	2BL	Brown-Guedira et al. (2003)
Lr52	*T. timopheevii* subsp. *viticulosum*	2A	Tar et al. (2008)
Lr54	*Ae. kotschyi*	2DL	Marais et al. (2005)
Lr59	*Ae. peregrina*	1AL	Marais et al. (2008)
Lr57	*Ae. geniculata*	5DS	Kuraparthy et al. (2009)
Lr58	*Ae. triuncialis*	2BL	Kuraparthy et al. (2011)
Lr62	*Ae. neglecta*	6AS	Marais et al. (2009)
Lr56	*Ae. sharonensis*	6A	Marais et al. (2010)
Lr25	*Secale cereale*	4BL	Procunier et al. (1995) Singh et al. (2011)
Lr26		1BL	Mago et al. (2005a)
Lr44	*T. spelta*	1B	Dyck and Sykes (1994)
Lr37	*Ae. ventricosa*	2AS	Helguera et al. (2003)
Lr38	*Ag. intermedium*	2AL	Mebrate et al. (2008)
Lr19	*Ag. elongatum*	7DL	Prins et al. (2001) Gupta et al. (2006)
Lr24		3DL	Dedryver et al. (1996) Gupta et al. (2006)
Lr29		7DS	Procunier et al. (1995)
Stripe rust resistance genes			
Yr37	*Ae. kotschyi*	2DL	Heyns et al. (2011)
Yr38	*Ae. sharonensis*	6AL	Marais et al. (2010)
Yr40	*Ae. geniculata*	5DS	Kuraparthy et al. (2009)
Yr42	*Ae. neglecta*	6AS	Marais et al. (2009)
Yr8	*Ae. comosa*	2A, 2D	Riley et al. (1968)
Yr5	*T. spelta*	2BL	McGrann et al. (2014)
Yr17	*Ae. ventricosa*	2AS	Robert et al. (1999)
Yr70	*Ae. umbellulata*	5DS	Bansal et al. (2016)
Yr9	*S. cereale*	1BL/1RS	Mago et al. (2005a)
Yr50	*Th. intermedium*	4BL	Liu et al. (2013)
Yr69	*Th. ponticum*		
Sr2	*T. turgidum*	3BS	Mago et al. (2011)
Sr13		1DL/1RS	Simons et al. (2011)
Sr34	*Ae. comosa*	2A, 2D	Friebe et al. (1996)
Sr36	*T. timopheevii*	2BS	Tsilo et al. (2008)
Sr40		2BS	Wu et al. (2009)

(continued)

Table 6.2 (continued)

Gene	Source	Chromosome	Reference
Sr37		4BL	Zhang et al. (2012)
Sr38	*Ae. ventricosa*	2AS	Helguera et al. (2003)
Sr53	*Ae. geniculate*	5DL	Liu et al. (2011)
Sr27	*Secale cereale*	3AS	Singh et al. (2011)
Sr31		1BL	Mago et al. (2002)
Sr50		1DL/1RS	Anugrahwati et al. (2008)
Sr24	*Th. elongatum*	3DL	Mago et al. (2005)
Sr25		7DL	Liu et al. (2010)
Sr26		6AL	Mago et al. (2005)
Sr43		7DL	Xu et al. (2009)
Sr44	*Th. intermedium*	7DS	Liu et al. (2013)
Sr52	*Dasypyrum villosum*	6AL	Qi et al. (2011)
Sr53	*Ae. geniculata*	5DL	Liu et al. (2011)
Sr59	*Secale cereale*	-	Rahmatov et al. (2016)
Powdery mildew resistance genes			
Pm5a	*T. dicoccum*	*7BL*	Law and Wolfe (1966)
Pm49		*2BS*	Piarulli et al. (2012)
Pm50		*2AL*	Mohler et al. (2013)
Pm1d	*T. spelta* var. *duhamelianum*	7AL	Hsam et al. (1998)
Pm4b	*T. carthlicum*	2AL	The et al. (1979)
Pm33		2BL	Yi et al. (2008)
Pm3b	*T. sphaerococcum*	1AS	Yahiaoui et al. (2004)
Pm7	*S. cereale*	4BL	Friebe et al. (1994)
Pm20		6BS	Heun et al. (1990)
Pm8		1BL/1RS	Mohler et al. (2001)
Pm17		1BL/1RS	Mohler et al. (2001)
Pm29	*Ae. ovate*	*7DL*	Zeller et al. (2002)
Pm13	*Ae. longissima*	*3B*	Cenci et al. (1999)
Pm33	*Ae. umbellulata*	*2BL*	Zhu et al. (2005)
Pm6	*T. timopheevii*	*2B*	Jorgenson (1973)
Pm27		*6B*	Jarve et al. (2000)
Pm37		*7AL*	Perugini et al. (2008)
Pm40	*Th. intermedium*	*7BS*	Luo et al. (2009)
Pm43		*2DL*	He et al. (2009)
Pm51	*Th. ponticum*	*2BL*	Zhan et al. (2014)
Pm21	*Haynaldia villosum*	*6AS*	Qi et al. (1996)
Pm55	*Dasypyrum villosum*	*5AS*	Zhang et al. (2016)
Pm56	*Secale cereale*	*6AS*	Liu et al. (2017)
Pm57	*Ae. searsii*	*2BL*	Liu et al. (2017a)
Fusarium head blight resistance genes			
FHB3	*Leymus racemosus*	*7AS*	Qi et al. (2008)

(continued)

Table 6.2 (continued)

Gene	Source	Chromosome	Reference
FHB6	*Elymus tsukushiensis*	*1AS*	Cainong et al. (2015)
FHB7	*Thinopyrum ponticum*	–	Wang et al. (2014a)
Drought tolerance genes			
KNAT3	*Agropyron elongatum*	–	Placido et al. (2013)
SERK1	*Agropyron elongatum*	–	Placido et al. (2013)

6.4.1 Rust Resistance

Rusts are one of the most common and serious diseases of wheat, and India witnessed several rust epidemics (Tomar et al. 2014). Three rusts, namely, stem or black rust caused by *Puccinia graminis* f. sp. *tritici* West, leaf or brown rust caused by *Puccinia triticina* Eriks, and stripe or yellow rust caused by *Puccinia striiformis* f. *Tritici* Eriks, are the most common diseases.

Stem rust (Sr) is a major disease of wheat grown in south, central, and peninsular India. The first rust epidemic was of stem rust which happened in 1786 A.D. in central India (Nagarajan and Joshi 1975). There are several reports of losses due to the epidemics of stem rust. At the international level also, stem rust is the most significant threat to global wheat production (Singh et al. 2008) with the emergence of Ug99 group of races. Since 1950 stem rust is effectively controlled by successful deployment of resistance genes from wild and cultivated wheats. The emergence of UG99 in East Africa in 1998–1999 and its spread thereafter posed serious threat to world wheat production. Wild wheat are rich source of Sr resistance gene, and till date many Sr resistance genes have been introduced into cultivated wheat including *Sr24*, *Sr25*, *Sr26*, *Sr32*, *Sr33*, *Sr36*, *Sr37*, *Sr38*, *Sr39*, *Sr40*, *Sr44*, *Sr45*, *Sr47*, *Sr50*, *Sr51*, *Sr52*, and *Sr*53 contributing a lot in standing wheat against the disease.

Leaf rust (Lr) has a widespread occurrence in the country and is the most dynamic of all the rusts. Leaf rust occurrence in huge scale was observed during 1971–1973 in northern plains (Joshi et al. 1975) and during 1993–1994 in northeastern plain zone (NEPZ) (Nagarajan 2008). There are about 80 known *Lr* resistance genes of which resistance gene derived from wild germplasm includes *Lr9 and Lr76* from *Ae. umbellulata*; *Lr35*, *Lr36*, *Lr47*, *and Lr*51 from *Ae. speltoides*; *Lr37* from *Ae. ventricosa*; *Lr38* from *Th. intermedium*; *Lr28*, *Lr35*, *Lr36*, *Lr51*, and *Lr66* from *Ae. speltoides*; *Lr21*, *Lr22a*, *Lr32*, *Lr39*, *Lr40*, *Lr41*, and *Lr42* from *Ae. tauschii*; *Lr57* from *Ae. geniculata*; *Lr58* from *Ae. triuncialis*; and *Lr53*, *Yr15*, *Yr35*, and *Yr36* from *T. dicoccoides*, well known to effectively control the disease.

Stripe rust (YR) is destructive and important in the northern areas of India, where frequent epidemics have occurred. It can cause yield losses as high as 100% when under the condition found suitable for pathogen growth (Chen 2005). Although, it is a disease of cooler climate, new races tolerant to high temperature have recently been identified (Milus et al. 2009), which enhances the damage potential considerably. Among the 72 genes identified, 18 are adult-plant resistance genes, namely, *Yr11–Yr14*, *Yr16*, *Yr18*, *Yr29*, *Yr30*, *Yr34*, *Yr36*, *Yr39*, *Yr46*, *Yr48*, *Yr49*, *Yr52*,

Yr54, *Yr59*, and *Yr62*, whereas others are all-stage resistance genes. Of which about 14 resistance genes are derived from wild species such as *Aegilops tauschii*, the tetraploid emmer (*Triticum dicoccoides*), durum wheat (*Triticum durum and Triticum turgidum*), and hexaploid wheat (*Triticum. spelta* album), or from more distantly related species such as *Aegilops comosa*, *Aegilops kotschyi*, *Aegilops sharonensis*, *Aegilops geniculata*, *Aegilops neglecta*, *Secale cereale*, and *Thinopyrum intermedium.* Systematic work on race analysis and testing of varieties against wheat rusts in India started in 1931 (Mehta 1940, Vasudeva et al. 1962). Attempts to understand the genetic basis of rust resistance began in the 1960s. Growing cultivars resistant to rust is the most sustainable, cost-effective, and environmentally friendly approach for controlling rust diseases. To date, more than 300 rust resistance genes including 100 leaf rust, 90 stem rust, and 80 stripe rust have been identified of which 41 genes for leaf rust and 15 for stripe rust resistance have been transferred from wild relatives making wild contribution to nearly about 50 % in providing resistance to wheat. Wheat wide hybridization program at Punjab Agricultural University led to transfer and mapping of more than 20 leaf rust and stripe rust resistance genes from different progenitor and non-progenitor species. More than 150 accessions of *Ae. tauschii* collected from different parts of the world have been screened for leaf rust and stripe rust for more than 10 years, and resistant accessions have been identified. Molecular diversity analysis also indicated a considerable amount of diversity among this collection. One leaf rust resistance gene *LrT* have been mapped into chromosome 2DS by developing a mapping population from cross *Ae. tauschii* acc pau 14195/PBW114/WH542 (Fig. 6.1).

Leaf rust- and stripe rust-resistant introgression lines have been developed in hexaploid background by transferring resistance genes from four different species, viz., *Lr57-Yr40* from *Ae. geniculata* (UUMM), *Lr76-Yr70* from *Ae. umbellulata* (UU), *LrP-YrP* from *Ae. peregrina* (UUSS), and *LrAc-YrAc* from *Ae. caudata* (CC), and these genes have been mapped on chromosome 5DS after their respective independent transfer (Fig. 6.2).

6.4.2 Powdery Mildew (PM) Resistance

Powdery mildew, caused by the biotrophic fungus *Blumeria graminis* f. sp. *tritici* (*Bgt*), is a foliar disease occurring in cool, humid climates during the entire growth period of wheat. Its epidemic, particularly at the later growth stage, results in significant yield loss and grain quality deterioration. Powdery mildew occurs almost everywhere wheat is grown; its increased incidences are due to increased application of nitrogen fertilizer, which favors the disease. Powdery mildew is important in the cooler regions of China, Japan, and other areas in Asia, in North and East Africa, in northern Europe, and in eastern North America (Roelfs 1977, Saari and Wilcoxson 1974). It is also important in warmer, humid regions with mild winters where wheat is planted in the autumn, such as parts of the Southern Cone of South America and the Southeastern United States.

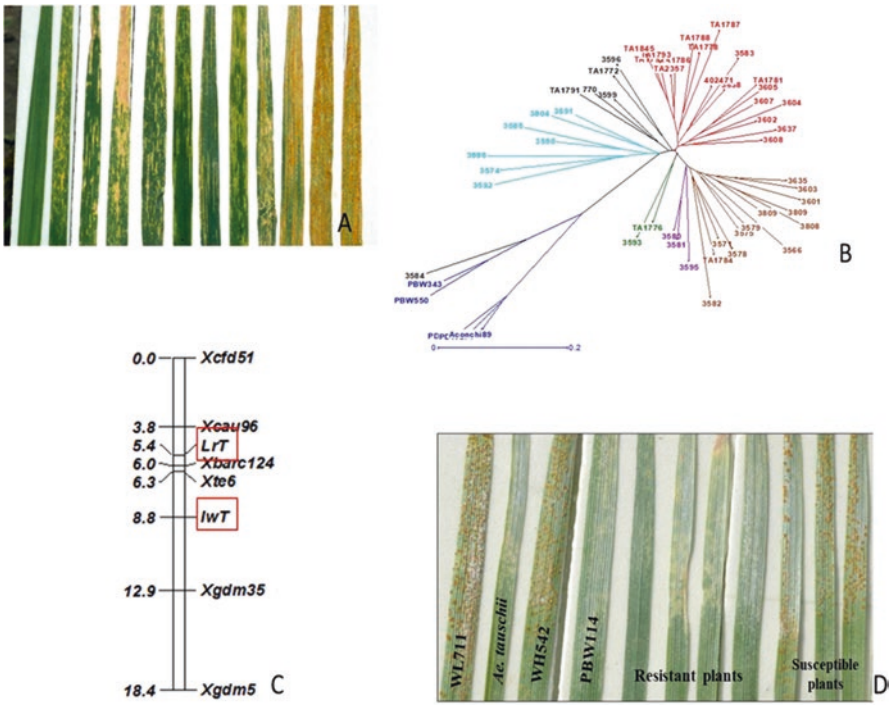

Fig. 6.1 (**a**) Variation in stripe rust reaction in different accessions of *Aegilops tauschii* collection at PAU. (**b**) Molecular diversity of 85 *Ae. tauschii* accessions with SSR markers. (**c**) Mapping of leaf rust resistance gene (*LrT*) and non-glaucous gene (*IwT*) in mapping population F_3 *Ae. tauschii* accpau 14195/PBW114/WH542, and (**d**) leaf rust reaction of parental lines—WL711, *Ae. tauschii* accpau 14195, WH542, and PBW114 along with resistant and susceptible plants in mapping population

In wheat, defense against powdery mildew is represented by two groups of genes: knockout susceptible gene represented by *MLO* genes and resistant genes represented by *Pm* genes. *MLO* genes are susceptibility genes and are present in three homologous copies on three wheat genomes (*TaMLO-A1*, *TaMLO-B1*, and *TaMLO-D1*), and their mode of action is reverse to that of resistance genes. These genes encode proteins to repress defenses response against *Pm* diseases and first reported in barley (Piffanelli et al. 2002). Durable resistance is achieved if there is mutation in all three *MLO* copies (Wang et al. 2014a). On the other hand, *Pm* resistance genes present mostly in single copy and interact according to Flor's gene-for-gene hypothesis with avirulent proteins of the pathogens to confer resistance to the diseases. At present, more than 70 formally designated *Pm* resistance genes *Pm1*–*Pm58* have been reported at 53 loci (McIntosh et al. 2017). Most of these resistance genes are major genes conferring race-specific resistance and are easily used in disease resistance breeding. Till date there are 55 designated powdery mildew resistance genes. Powdery mildew resistance genes from progenitor species includes

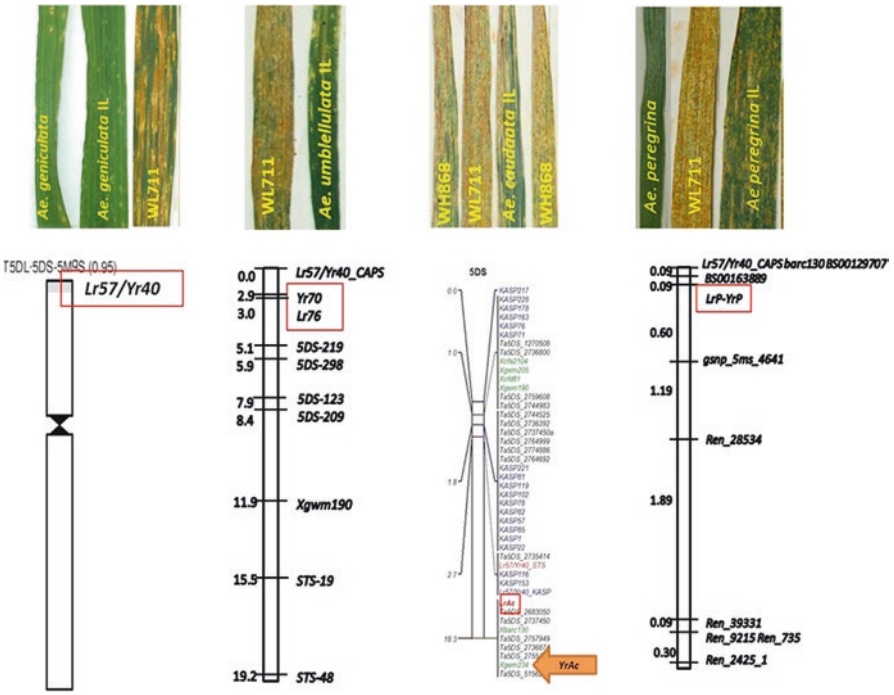

Fig. 6.2 Transfer and mapping of leaf rust and stripe rust resistance genes on chromosome 5DS of hexaploid wheat from four different non-progenitor species of *Ae. geniculata*, *Ae. umbellulata*, *Ae. caudata*, and *Ae. peregrina* (Ae., *Aegilops*; IL, introgression line)

Pm4a from *T. monococcum, Pm4b* from *T. carthlicum*, *Pm12* from *Aegilops speltoides*, *Pm13* from *Ae. longissima*, and *Pm25* from *T. monococcum* and from distant relative or non-progenitor species of wheat includes *Pm40* and *Pm43* from *Thinopyrum intermedium*, *Pm21* from *Haynaldia villosa*, *and Pm7*, *Pm8*, *Pm17*, *Pm20*, and *PmCn17* from *Secale cereale*.

6.4.3 Karnal Bunt (KB) Resistance

Karnal bunt (KB) also called partial bunt caused by heterothallic fungus *Tilletia indica* (syn. *Neovossia indica*) is an important seed-based disease. The disease was first reported in 1931 in Karnal, India (Mitra 1931). Since then it is widespread in Pakistan, Nepal, Iraq, Iran, Afghanistan, South Africa, Mexico, and the United States. Karnal bunt gained importance due to quarantine restriction imposed by more than 70 countries (Rush et al. 2005). Infected grains emit unpleasant fishy odor, and wheat containing more than 3% bunted kernels is considered unfit for human consumption (Warham 1986). The pathogen is soil, seed, and air borne in

nature and hence difficult to control once introduced and established in an area. Development of KB-resistant varieties is difficult due to limited variability for KB resistance in hexaploid wheat (Dhaliwal et al. 1993), quantitative nature of inheritance, and considerable influence of environment on screening for disease resistance (Dhaliwal and Singh 1997). A synthetic amphiploid between *T. durum* and a KB-resistant *Ae. tauschii* accessions has been generated to transfer resistance to hexaploid background (Chhuneja et al. 2008). Limited success has been achieved in KB resistance due to tedious screening methodology and complex nature of resistance. With genome sequencing of *T. indica* of size 26.7 Mb, putative genes involved in molecular pathogenesis will be identified and will help to identify host pathogen interaction and design novel biomarkers for pathogen detection.

6.4.4 Fusarium Head Blight (FHB) Resistance

Fusarium head blight (FHB), also called head scab caused by *Fusarium graminearum*, is an important disease of wheat but also infects barley, rye, triticale, and oats. Under extreme condition it may cause severe losses of seed quality and yield (Dexter et al. 1996, Bai and Shaner 2004, Steiner et al. 2004). The disease is more important in the United States, Canada, Europe, Asia, and South America. It was first reported in England in the year 1884 and in Nebraska in 1898. The disease causes premature bleaching of infected spikelets starting with white appearance of one or more, which on later stage led to production of orange, spore-bearing structures called sporodochia at the base of the glumes. The fungus may eventually kill the developing seed at about the soft dough stage. Wheat and barley infected by FHB may contain mycotoxins such as deoxynivalenol (DON) (Pestka 2010). The disease develops well in the area with high humidity and warm temperatures during the anthesis (Juroszek and von Tiedemann 2015). The most effective strategy for controlling FHB in wheat is through the development of resistant cultivars. Resistance to FHB exhibits quantitative variation, and its inheritance involves several loci on different chromosomes (Kolb et al. 2001). Genotype x environment interaction complicates the phenotypic evaluation of FHB resistance and makes screening of FHB resistance laborious, time consuming, and costly (Rudd et al. 2001).

FHB resistance is mainly controlled by major and minor QTLs and five types of resistance have been described (Shroeder and Christensen 1963, Mesterhazy 1995): type I resistance is against initial infection; type II resistance is against spread in infected tissue; type III resistance is against kernel infection; type IV resistance is against tolerance, and type V resistance is against accumulation of toxins. Because of the quantitative nature of resistance, development of resistant cultivars has been slow, and to date only a few cultivars with moderate resistance have been released worldwide. Efforts to breed for resistance using traditional and molecular techniques are continuing, and both native and exotic sources of resistance have been identified in the FHB-prone regions of the world (Bai and Shaner 2004).

For FHB nearly about 100 quantitative trait loci (QTL) were described to date by genetic mapping in diverse hexaploid wheat germplasm (Buerstmayr et al. 2009).

Of these *FHB1* on chromosome 3BS was conferring a type II resistance and one of the strongest and most reliable QTL (Waldron et al. 1999). Type II resistance conferred by *FHB1* is associated with its ability to inactivate DON into non-toxin DON-3-O-glucoside. Gunnaiah et al. (2012) suggested that the higher accumulation of phenylpropanoids is responsible for resistance to *FHB1*, while Xiao et al. (2013) observed changed jasmonic acid signaling is a possible mechanism for the resistance mediated by the QTL. Zhuang et al. (2013) suggested that a pectin methyl esterase inhibitor gene that is downregulated in susceptible lines could be the causal gene. He et al. (2016) identified major and minor QTLs for FHB resistance in synthetic hexaploid wheat Soru#1. Major QTL was found on chromosome 2DLc, explaining 15–22% of the phenotypic variation, while minor QTL was detected on chromosomes 2AS, 2DL, 4AL, 4DS, and 5DL. The mechanism of resistance of Soru#1 to FHB was mainly of type I for resistance to initial infection, conditioned by the major QTL on 2DLc and minor ones that often coincided with QTL for DH, PH, and anther extrusion (AE).

Kopahnke et al. (2009) screened 257 accessions of *Triticum monococcum*, 32 accessions of *T. dicoccum*, 27 accessions of *T. turgidum*, and 5 accessions of *T. boeticum* for FHB. Seven accessions of *T. monococcum* and six accessions of *T. dicoccum* were identified showing a high level of FHB resistance. Three accessions of *T. monococcum* have a haplotype different from *T. aestivum* accessions carrying the resistance allele at chromosome 5A. *T. dicoccum* and *T. spelta* genotypes were found to be moderately resistant to FHB as DON accumulation was significantly low in these as compared to modern common wheat varieties. FHB-resistant emmer and spelt wheat materials also have some outstanding grain quality parameters. Ban and Watanabe (2001) found that the 3A chromosome from the *T. dicoccoides* provided resistance to head bleaching after Fusarium inoculation. Stack et al. (2002) developed a single chromosome recombinant population for the 3A chromosome from Langdon *T. dicoccoides*-3A and identified a FHB resistance QTL on 3AS. Kumar et al. (2007) mapped a significant QTL to chromosome 7AL derived from the *T. dicoccoides* accession PI478742. In a mapping population derived from the cross of the *T. durum* cultivar Strongfield with the *Triticum carthlicum* cultivar Blackbird, two significant QTL for FHB spread were found, one from each of the two parents (Somers et al. 2006) on chromosome 2BS and 6BS, respectively.

Triticum monococcum, *T. dicoccom*, *T. polonicum*, *T. spelta*, and *T. aestivum* were tested for FHB resistance, and *T. spelta* have the strongest response with lowest levels of mycotoxins, while *T. polonicum* had weakest response with high level of mycotoxins. Steed et al. (2005) identified and mapped *T. macha*-derived QTL for type I resistance on the short arm of chromosome 4A co-segregating with *Xgwm165*. Shen et al. (2004) identified a QTL for FHB resistance from *Thinopyrum ponticum*-derived disomic substitution lines for chromosome 7el(7D). The QTL mapped to the distal region on the long arm of the 7el chromosome contributing resistance to fungal spread. Several further alien species that have potential as donors of FHB resistance genes have not been genetically mapped so far, like *Elymus humidas*, *Elymus racemifar*, *Roegneria kamoji*, and *Leymus racemosus* (Oliver et al. 2005). To incorporate *L. racemosus* FHB resistance in wheat, several addition, substitution, and translocation lines were generated by Chen et al. (2005).

6.4.5 *Wheat Blast Resistance*

Wheat blast caused by filamentous, ascomycetous fungus *Pyricularia oryzae* (*Magnaporthe oryzae*) is divided into host-specific pathotypes with *Oryza* isolate affect *Oryza* spp., *Setaria* isolate affect *Setaria* spp. (Foxtail millet), *Eleucine* isolate on *Eleucine* spp. (finger millet) with *Loliumisiolate* for rye grass, and *Triticum* isolate on wheat. Blast has emerged as an explosive threat to wheat production that can cause up to 100% yield losses under conducive environmental conditions. First incidence of blast was observed in 1985 in the Brazilian state of Paraná and thereafter in other important wheat-producing states of Brazil (Picinini and Fernandes 1990), while in 2016, a wheat blast outbreak was reported for the first time outside of South America and Bangladesh. Bangladeshi wheat blast fungus was similar to as found in South America (Islam et al. 2016). Blast-infected wheat plants lead to small, shriveled, and deformed grains with low test weight (Goulart et al. 2007). Highest yield losses up to 100% occur when spike infections begin during flowering or early grain formation (Goulart et al. 2007) in susceptible cultivars. Blast appears as bleaching of the spike leading to partial or total spike sterility depending on susceptibility of cultivar, timing, and point of infection.

Resistance of blast in wheat follows a gene-for-gene relationship (Anh et al. 2015). Several physiological races of pathogen have already been identified within pathogen populations (Urashima et al. 2004, Maciel et al. 2014, Cruz et al. 2016). It has also been reported that spike blast and leaf blast in wheat plant may be controlled by different resistance mechanisms (Cruz et al. 2012, Maciel et al. 2014). Qualitative resistance is more commonly found at the seedling stage (Maciel et al. 2014). Till now eight blast resistance genes have been reported in wheat of which only two *Rmg7* and *Rmg8* have been reported from tetraploid wheat relative *T. dicoccoides* (Cumagun et al. 2014), while the rest are from hexaploid cultivars. Cruz et al. (2016) identified a wheat head blast resistance trait contained on a wild wheat chromosome segment (the 2NS translocation segment from *Aegilops ventricosa*) conferring head blast resistance under natural epidemic conditions. Resistance to blast has also been reported from synthetic wheat (Cruz et al. 2010).

6.5 Abiotic Stress Resistance from Wild Germplasm of Wheat

Abiotic stresses like frost, cold, drought, heat, and salinity severely limit plant growth and development as well as affect morphological, biochemical, and physiological characters limiting the final yield in wheat. High temperatures, low temperature drought, and salinity represent stress factors associated with plant cell dehydration (Tables 6.1 and 6.2).

6.5.1 Salt Tolerance

About 8–10% of the wheat area planted under irrigated and rain-fed conditions in India, Pakistan, Iran, Egypt, Libya, and Mexico is affected by salinity (Mujeeb-Kazi and Diaz de Leon 2002). There is a need to improve salt tolerance of wheat in order to utilize salt-rich area for growing of wheat. Intensive efforts have been made to search for salt-tolerant genes/alleles in wheat wild relatives (Nevo and Chen 2010). Australian scientists have produced a salt-tolerant commercial durum wheat variety by introducing an allele *TmHKT1;5-A* from its wild relative, *Triticum monococcum*, via crossbreeding. This cultivar showed 25% greater yield in high-saline fields compared to its Tamaroi parent (Munns et al. 2012, James et al. 2006). *TmHKT1;5-A* reduces the Na^+ level in plant leaves that prevents yield losses under salinity stress. Gorham et al. (1991) reported that *T. urartu* shows greater Na^+ exclusion and K^+/Na^+ discrimination than durum wheat (AABB), as do the closely related A-genome species *T. monococcum* ssp. *monococcum* and *T.monococcum* ssp. *aegilopoides* (syn. *T.boeoticum*). Different accessions of *Triticum boeoticum, T. monococcum*, and *T. urartu*, when grown in 50 $molm^{-3}$ NaCl+2.5 and $molm^{-3}$ $CaCl_2$ (Gorham et al. 1991), showed increased potassium concentrations in leaves as compared to *T. durum* grown under the same conditions indicating the A genome of modern tetraploid wheats is entirely different from its progenitor species.

Aegilops tauschii exhibits high Na+ exclusion (Schachtman et al. 1991) and is able to grow in 150 mM NaCl without showing any visual symptoms of salinity stress (Shavrukov et al. 2009). The *Ae. tauschii* accession study also showed a moderate range of sodium accumulation in the shoot. A number of accessions of *Ae. tauschii*, with a wide range of Na^+ exclusion traits, have been identified (Schachtman et al. 1991). Gorham (1990) identified K/Na discrimination in *Ae. tauschii*, *T. araraticum*, *and T. dicooccum.* Thus wild wheat is important for transfer of this character to hexaploid wheat. Some accessions of *T. turgidum* ssp. *dicoccoides* from Israel found to have lower rates of Na^+ uptake than the durum (Nevo et al. 1992), when tested by applying $^{22}Na^+$ to seedlings. Gilboa accession of *T. dicoccoides* is found to have higher tolerance than in cultivated durum wheat. Thus *T. dicoccoides* harbors genetic resources for salinity tolerance that can be used in wheat improvement. This was reinforced in a later study (Nevo et al. 1993) when wild emmer wheat genotypes from eastern Samaria steppes were found to be ripening in the presence of 250 mM NaCl (about 40% of sea water).

6.5.2 Cold Stress Tolerance

Cold especially in areas of high altitude presents one of the major limitations for plant growth and yield worldwide, due to its negative effects on plant physiology, biochemistry, and molecular biology. Plant cell response to dehydration under cold stress includes an accumulation of osmotically active compounds including proteins

such as dehydrins. Wang et al. (2014a) have identified 54 dehydrin unigenes in common wheat genome by a search of wheat EST database. Mostly studies on cold tolerance have been done in hexaploid wheat, and very few alleles have been incorporated from wild wheats. Frost tolerance is determined by major loci (*Fr*) located on the long arms of homologous group 5 of chromosomes. *Fr-A1* and *Fr-A2* were mapped on chromosome 5AL in close proximity to the gene *Vrn-A1* in *T. monococcum*. *FrA1* is at distance of 30cm from *FrA2*. Another gene *Cbf1* which is a cold-regulated CBF like barley gene has also been mapped near to *FrA2* in *T. monococcum* (Va´gu´jfalvi et al. 2003)

6.5.3 Drought Tolerance

Development of higher-yielding crops under water-limited environments is the most viable solution to stabilizing and increasing wheat production under changing climatic conditions. Exploitation of drought-tolerant wild species along with high-throughput genomic technologies resulted in dissection of molecular aspects of drought tolerance.

Wild emmer wheat is important for its high drought tolerance, and some of *T. dicoccoides* genotypes are fully fertile in arid-desert environments. Wild emmer wheat accessions were shown to thrive better under water-limited conditions in terms of their productivity and stability compared to durum wheat. *T. dicoccoides* is an important source of drought-related gene and is highly suitable as a donor for improving drought tolerance in cultivated wheat species.

Lucas et al. (2011) cloned *TdicTMPIT1* (integral transmembrane protein inducible by tumor necrosis factor-α, TNF-α) associated with the drought stress response from wild emmer root tissue causing an increased levels of expression upon osmotic stress. Lucas et al. (2011a) cloned *TdicDRF1* (DRE binding factor 1) from wild emmer wheat. Its DNA-binding domain, AP2/ERF, was shown to bind to drought-responsive element (DRE). Mohamed et al. (2010) screened 16 wild tetraploid wheat accessions and two common wheat, and different drought stress-related traits were measured like damage to the thylakoid membranes, flag leaf temperature depression (FLTD), and spike temperature depression (STD) during exposure to heat stress for 16 days post-anthesis (DPA). This study identified potential heat-tolerant wild tetraploid wheat germplasm that can be incorporated into wheat breeding programs to improve heat tolerance in cultivated common and durum wheat.

6.5.4 Heat Stress Tolerance

Since the average daily temperature is rising globally, plants respond to this as stress by reducing their end products/yield. Optimum temperature for wheat crop during the post-anthesis period is 22–25°C; beyond that the heat is felt, causing irreversible

damage by high temperature (Farooq et al. 2011). High temperature affects almost all the developmental stages of plant growth right from seedling to reproductive, but some pre-flowering and anthesis stages are relatively more sensitive as it causes deformation in flowering and leads to poor pollen viability (Cossani and Reynolds 2012). The effect of high temperature is also severe during grain filling due to the malformed and shriveled grains causing decreased grain weight. It has been reported that each °C rise in temperature above cardinal causes reduction in grain filling duration by 2.8 days (Streck 2005), grain numbers by 4% (Fischer 1985), gain weight by 5% (Tashiro and Wardlaw 1989), and grain yield by 3–4% (Wardlaw et al. 1989). The heat stress can cause losses up to 40 % (Hays et al. 2007). Heat tolerance is a quantitative trait, controlled by a number of genes/QTLs (Bohnert et al. 2006).

Populations of wild species frequently harbor high intraspecies variation for tolerance traits that are superior to what is available in the modern cultivars (Geng et al. 2016). *Triticum dicoccoides* and *T. monococcum* have been reported as potential sources of germplasms that can be used to enhance heat tolerance in bread wheat. Additionally, variable degrees of heat tolerance were observed in *Aegilops speltoides*, *Ae. longissima*, and *Ae. searsii* (Pradhan et al. 2012). However, only a small portion of the reported genetic variation in heat tolerance has been utilized due to limitations of conventional breeding methods.

Zaharieva et al. (2001) studied a collection of 157 *Aegilops geniculata* accessions with different ecogeographical origin and identified potential value of *Aegilops geniculata* for improvement of high temperature and drought stress tolerance in wheat. Accessions originating from harsh environments had high water-use efficiency with low chlorophyll content which limits the energy load from strong sunlight. Accessions originating from mild Mediterranean climate are characterized with high chlorophyll content, leaf area, and biomass production; thus thermal regulation of the leaf depends on transpiration.

Waines (1994) identified ten wild accessions of *Ae. speltoides*, *Ae. longissima*, and *Ae. searsii* as heat tolerant as these accessions showed normal vegetative and reproductive development under heat stress. *Ae. speltoides* the most probable B-genome donor of wheat has been observed to have displayed high level of resistance to major wheat diseases and has been found to be highly tolerant to terminal heat stress. A set of 800 backcross introgression lines of *Aegilops speltoides* have been developed in *Triticum durum* background. Genotyping of a set of 450 lines identified almost complete genome of *Ae. speltoides* in the form of small introgressed segments with multiple introgressions. These lines were phenotyped for three consecutive seasons, and QTL controlling yield component traits under heat stress were identified on chromosomes 2B, 3A, 3B, 5A, 5B, and 7A.

Gupta et al. (2010) evaluated 129 accessions of *Aegilops tauschii* Coss., the D-genome donor of wheat, for cell membrane stability (CMS) and TTC (2,3,5-triphenyl tetrazolium chloride)-based cell viability. A cell thermotolerance index was calculated which identified ten tolerant *Ae. tauschii* genotypes. Recurrent selection has been done for improving wheat yield using ancestor *T. tauschii* as a gene donor, leading to increased rates of grain filling and larger grains in BC_1F_6 plants (Gororo et al. 2002).

Pradhan et al. (2012) screened 52 accessions belonging to five *Aegilops* species for heat stress tolerance and identified that heat stress decreased chlorophyll, grain number per spike, individual grain weight, and grain yield per plant in all the species. But based on grain yield, *Ae. speltoides* Tausch and *A. geniculate* Roth were the most tolerant, and *A. longissima* Schweinf. and Muschl. was highly susceptible to HT stress.

6.6 Genomic Tools to Transfer Useful Traits from Wild Species to Cultivated Wheat

6.6.1 Genotyping by Sequencing (GBS)

Genotyping by sequencing, or next-generation genotyping, is a genetic screening method for discovering novel plant and animal SNPs and performing genotyping studies. It provides a lower-cost alternative to arrays for studying genetic variation. The advantages includes:

1. Multiple samples can be sequenced at predetermined region of genetic variation.
2. It provides low cost per sample and reduction of ascertainment bias compared with SNP chips. 3. Other types of variants can be discovered like deletions, insertions, and microsatellites in addition to SNPs.
3. GBS data analysis does not require reference genome sequence.
4. It can be used efficiently for genetic mapping, association mapping, construction of haplotype maps, and testing of pure lines as well as for genomic selection for plant studies (Poland et al. 2012, Poland and Rife 2012).

Genotyping by sequencing is cost-effective for populations with complex genomes or limited available of resources. Genotyping by sequencing is a simple highly multiplexed system for constructing reduced representation libraries for the Illumina NGS platform developed in the Buckler lab (Elshire et al. 2011). GBS techniques also include amplicon-based targeted sequencing and hybridization-based enrichment sequencing. It generates large numbers of SNPs for use in genetic analyses and genotyping (Beissinger et al. 2013). Briefly, the technology includes extraction of high-quality DNA and DNA quantification and normalization followed by restriction digestion and ligation of adaptors containing barcodes (barcode region of adapter will be different for each DNA sample), these restricted fragments represent GBS library, and the fragments are then sequenced on next-generation sequencing platforms followed by bioinformatics pipeline for GBS data analysis. A two-enzyme (*PstI/Msp*I) GBS protocol, which provides a greater degree of complexity reduction and uniform library for sequencing than the original protocol using *Ape*KI, has been developed and applied to wheat (Poland et al. 2012). GBS has been used to study for mapping of genes in wild wheat species.

GBS was used to identify the loci conferring resistance to stem rust pathogen races including Ug99 in an *Aegilops umbellulata* biparental mapping population. A genetic linkage map, comprised of 1993 SNP markers, was created for all the seven chromosomes of *Aegilops umbellulata* using GBS. A major stem rust resistance QTL explaining 80% and 52% of phenotypic variations for TTTTF and TTTK, respectively, was detected on chromosome 2U of *Aegilops umbellulata*. The tightly linked markers to the novel genes identified can be used to transfer in commercial wheat varieties (Edae et al. 2017).

6.6.2 SNP Chips

SNP array is a type of DNA microarray which is used to detect polymorphisms at a single nucleotide site. SNP variation is the most frequent type of variation in the genome. A DNA chip is a small piece of silicon glass (~1 cm^2) to which a large number of synthetic, single-stranded DNA oligonucleotides have been chemically bonded. Oligos function as DNA probes, and they anneal selectively only to those DNA molecules whose nucleotide sequences are exactly complementary. With the development of next-generation sequencing (NGS) technology and low-cost genome sequencing, a large number of SNPs are being identified and used to design arrays for major crops. SNP-based platforms are attractive genotyping tools because of reduced computational requirements for downstream data processing, high call frequency, low error rate, and ease of use (Wang et al. 2014b). Though SNP arrays are prone to ascertainment bias caused by preselection of SNPs in limited population sizes (Albrechtsen et al. 2010). High-density SNP arrays have been developed for a number of economically important crops, for example, 44K and 50K SNP arrays have been developed in rice (Singh et al. 2015), and 55K SNP chip (Cheng et al. 2017) has been developed in maize. SNP arrays comprise loci with unique positions along chromosomes or genomes, thereby largely avoiding the confusion associated with multiple sequence variants, especially in wheat having three genomes. In wheat, 9K SNP chip was the first SNP array platform available to wheat community in 2013 that is used to detect genomic regions targeted by breeding and improvement selection in wheat. For designing Illumina 9K iSelect assay, 9000 gene-associated SNPs were selected from Roche 454 sequence reads from nine wheat accessions originating from Australia, the United States, and Mexico. The sequence reads were assembled into 477,291 reference transcripts (RTs) and used to identify 25,454 SNPs with a validation rate of 85–90%. The distribution of alleles at the SNP sites was assessed using deep-coverage Illumina sequence data generated from a sample of 20 diverse wheat cultivars from Australia, China, Mexico, and the United States, 3 of which overlapped with the panel of 9 accessions used for 454 sequencing. Six hundered and fifty five SNPs were also discovered in a diverse panel of wheat landraces (27) and SNPs identified from a sequence capture assay of 3,500 genes in the parents of the SynOp mapping population (Cavanagh et al. 2013). In total, 7504 polymorphic loci were positioned on consensus map

using seven mapping populations including six biparental populations and one four-parent Multiparent Advanced Generation Inter-Cross (MAGIC) population. Another Illumina-based SNP platform is the wheat 90K SNP array which was made available in 2014 (Wang et al. 2014b); it provides dense coverage of wheat genome covering 90,000 gene-associated SNPs. The distribution of the 90K SNPs across populations was assessed in diverse panel of 726 accessions including tetraploid and hexaploid landraces. A total of eight biparental double haploid mapping populations were used to order SNPs; in total 46,977 SNP markers were genetically mapped. However, while there is ample amount of hexaploid SNP resources available, the majority of SNP markers developed to date are not suitable for use in wide crosses. The high level of sequence polymorphism between hexaploid wheat and its wild relatives makes it difficult to design polymerase chain reaction (PCR) primers for array-based probes (Winfield et al. 2016). To overcome this limitation, ultrahigh-density Affymetrix Axiom® SNP array of 820 K has been developed. To characterize significant proportion of the wheat genome, sequence capture targeted re-sequencing approach was used (Winfield et al. 2012), which was then used to identify large numbers of exome-specific SNPs (Allen et al. 2013). In this array, exome-captured sequences from a range of species, including members of the secondary and tertiary gene pool, which are a potential source of novel alleles suitable for introgression into the hexaploid genome were also incorporated. The resulting captured sequences were analyzed to identify a large number of putative SNPs between different varieties of hexaploid wheat and between hexaploid wheat and related species, including its putative progenitor species (*Aegilops tauschii*, *Aegilops speltoides*, and *Triticum urartu*) and various wild relatives. To evaluate the large-scale validation of the putative SNP markers, Axiom high-density genotyping platform (Affymetrix Inc., Santa Clara, CA) was used. The SNP markers and the Axiom genotyping array described here have resulted in the generation of a large number of validated varietal and species-specific SNPs which can be used to monitor and map introgressions within the hexaploid wheat genome. The capture and targeted re-sequencing of the wheat exome, ~900 million sequences from 43 bread wheat accessions and wheat relatives, were generated. These included 14 diploid species including A-, B-, and D-genome progenitors as well as representatives of E, J, R, and T genomes, five tetraploids (AB and AG), 23 hexaploids, and 1 decaploid. Of the sequences generated, 344.5 million (38%) could be mapped back to sequences on the array. In total 56 505 markers were mapped to the 21 wheat chromosomes in three mapping populations. Though this array can contribute toward high-throughput genotyping, it is not cost-effective. In addition, the majority of the markers on this array was designed to genotype polymorphisms between wheat and its near relatives and progenitors and hence is of limited direct value to wheat breeders who are specifically interested in comparing hexaploid germplasm (Allen et al. 2017). To overcome these limitations, a set of 35 143 informative markers were identified from the data obtained from 820K wheat array. A 384 microplate format Axiom® array (also to be called the Wheat Breeders' Array) was designed to confirm the utility of the selected SNP markers. The Wheat Breeders' Array was synthesized for high-throughput sample screening and rapid downstream data analysis. In addition

to these arrays, a new array has recently been designed by the Chinese Academy of Agricultural Sciences and synthesized by Affymetrix. The Wheat 660 is generated from screening four 660K SNP arrays against 192 wheat accessions including 60 worldwide modern varieties, 72 landraces, 30 wild emmer wheat, and 30 *Ae. tauschii*. It can detect more than 630k (630,000) SNPs in wheat. The Wheat 660 has the following advantages: all the SNPs are genome specific with clear genotypic profiles which makes accurate automatic reading possible. It has the highest density in wheat up to now, and since the 630K SNP was designed in one chip, therefore the cost is much lower. All of the SNPs have been tested and confirmed; therefore it is highly efficient. Further, the SNPs on subgenome D is much more than the previously arrays and thus can meet the requirement for detection of D-genome diversity. The Wheat 660 array has a wide range of possible application. More than 90% of the SNPs are polymorphic among the wheat cultivars assessed. Therefore, the array can be used for gene discovery, haplotype mapping, genomic selection, and evolutionary studies. There are many examples available in literature regarding the use of SNP arrays in genome-wide association studies in wheat breeding lines (Cavanagh et al. 2013, Maccaferri et al. 2015), regarding mapping of disease resistance genes in wheat cultivars (Kertho et al. 2015, Wu et al. 2017), but only one or two case studies are there illustrating the use of SNP arrays for the mapping of genes transferred from wild species. Wang et al. in 2018 did molecular mapping of a stripe rust resistance gene *YrTZ*2 in wild emmer accession (TZ-2) using Infinium 90K iSelect SNP genotyping assay. *YrTZ*2 obtained from wild accession TZ-2 confers near-immunity resistance against prevailing *Pst* races in China. A set of 200 RILs obtained from cross between susceptible durum wheat cultivar Langdon and TZ-2 was used for stripe rust evaluation. Genetic analysis indicated that *YrTZ*-2 is controlled by single dominant gene. Using SSR and SNP markers from 90K iSelect assay, *YrTZ*2 was mapped into an 0.8 cM interval between SNP locus IWB19368 and SSR marker *Xgwm*413 and co-segregated with SNP locus IWB28744.

6.6.3 RNA Sequencing

RNA sequencing is the sequencing of transcriptome of the cell using next-generation sequencing platforms. It is also called as whole transcriptome shotgun sequencing (Morin et al. 2008). This technology enables the study of the genes expressed in a genome in unit time and in particular tissue type (Chu and Corey 2012). Specifically, RNA-Seq facilitates the ability to look at alternative gene spliced transcripts, post-transcriptional modifications, gene fusion, mutations/SNPs and changes in gene expression over time, or differences in gene expression in different groups or treatments (Maher et al. 2009). Briefly, the methodology in RNA sequencing included isolation of total RNA followed by isolation of mRNA, synthesis of cDNA from mRNA, and then sequencing of cDNA on next-generation platform followed by analysis. At Punjab Agricultural University, pathogen induced differential gene expression of leaf rust resistance gene *Lr57* was characterized in a near isogenic line

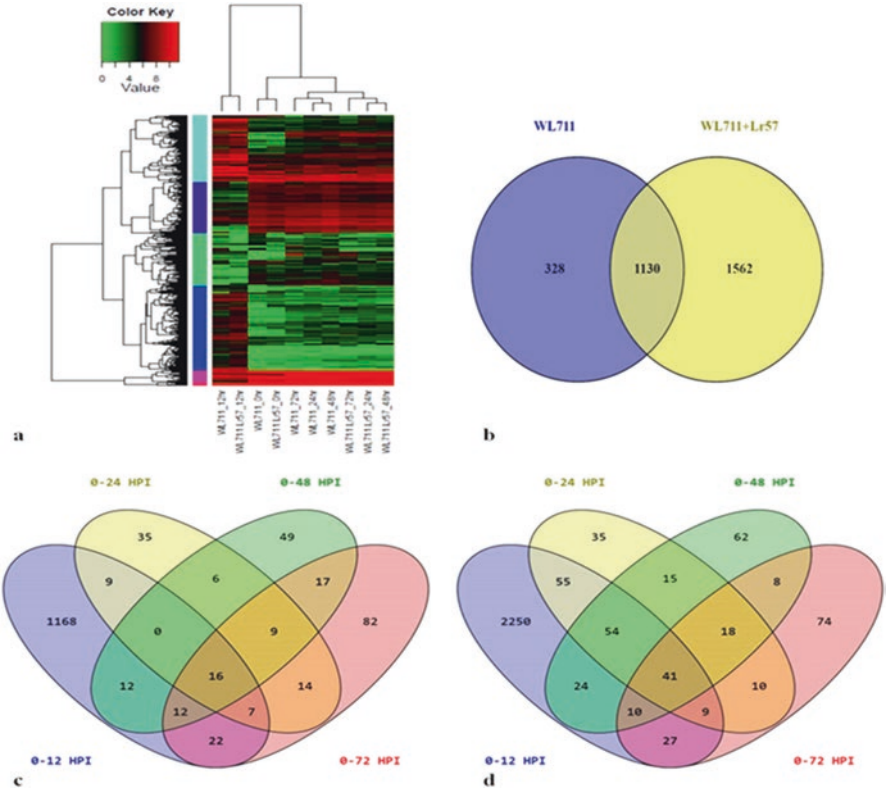

Fig. 6.3 (**a**) Expression profiling of differentially expressed genes in both WL711 and WL711+*Lr57*. Horizontal row represents the gene and vertical columns denote samples. (**b**) Venn diagram shows the distribution of differentially expressed genes in WL711 and WL711+*Lr57*. (**c**) Distribution of differentially expressed genes at different time points postinoculation in WL711. (**d**) Distribution of differentially expressed genes at different time points postinoculation in WL711+*Lr57* (Yadav et al. 2016)

carrying resistance gene (WL711+*Lr*57), and its susceptible recipient genotype WL711 (Fig. 6.3) RNA samples were collected at five different time points 0, 12, 24, 48, and 72 h postinoculation (HPI) with Pt77-5. WL711+*Lr57* showed much higher number of differentially expressed nucleotide-binding and leucine-rich repeat genes and expressed more protein kinases and pathogenesis-related proteins such as chitinases, glucanases, and other PR proteins as compared to susceptible genotype. Comparative analysis of the differentially expressed transcripts led to the identification of some transcripts which were specifically expressed only in WL711+*Lr57*. It was apparent from the whole transcriptome sequencing that the resistance gene *Lr57* directed the expression of different genes involved in building the resistance response in the host to combat invading pathogen (Yadav et al. 2016).

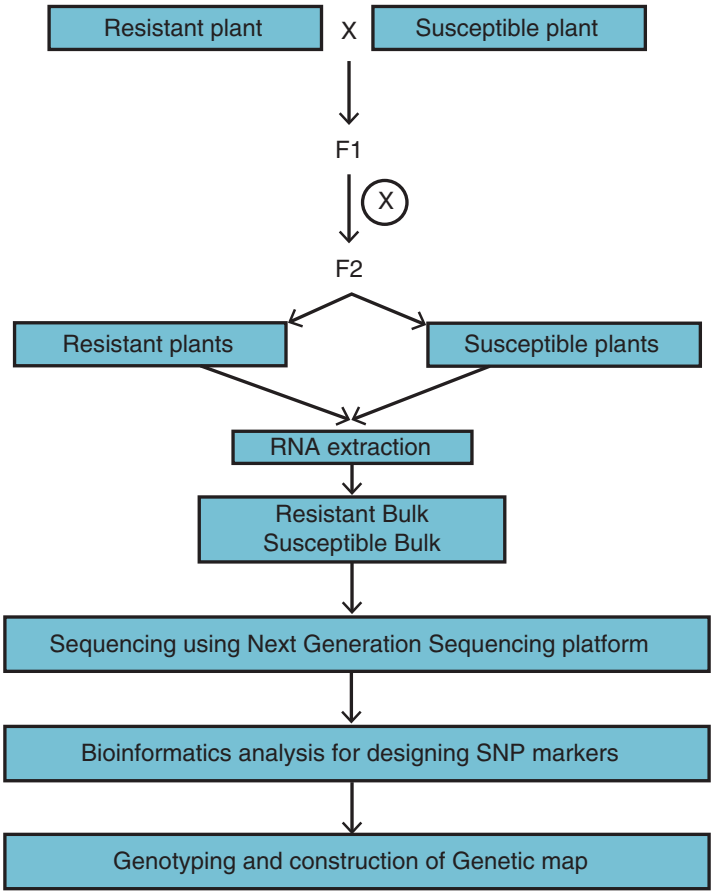

Fig. 6.4 RNA sequencing used for the mapping of genes

In wheat, RNA sequencing coupled with bulk segregant analysis (Michelmore et al. 1991) had been used for mapping of stripe rust resistance gene *Yr15*, a gene derived from Israeli wild emmer wheat *Triticum dicoccoides* (Ramirez-Gonzalez et al. 2015). Briefly, they generated the F_2 population from the cross between *Yr15* resistant and susceptible cultivar from three individual F_1 plants. Three resistant and three susceptible bulks were prepared from F_2 plants. Sequencing was done on Illumina HiSeq 2000 platform. The sequenced reads generated from parents as well as bulks were aligned against NCBI UniGene build and UCW gene models. Bulk frequency ratios were calculated to find out the SNPs in resistant and susceptible bulks. KASP markers were synthesized and validated on the F_2 population. A brief methodology of RNA sequencing used for the mapping of genes is depicted in Fig. 6.4.

6.6.4 *RenSeq (Resistance Gene Enrichment Through Sequencing)*

RenSeq is a NB-LRR (nucleotide-binding site-leucine-rich repeat) gene targeted, resistance gene enrichment, and sequencing method that enables the discovery and annotation of pathogen resistance gene family members in plant genome sequences (Fig. 6.5). RenSeq was successfully applied to the sequenced potato *Solanum tuberosum* clone DM and increased the number of identified NB-LRRs. RenSeq was also used to rapidly identify molecular markers that co-segregate with a pathogen resistance trait of interest. The technology was successfully applied to identify the markers that co-segregate with resistance toward the late blight pathogen *Phytophthora infestans* in two independent segregating populations (Jupe et al. 2013). Further, RenSeq was modified to MutRenSeq technology which is a three-step method that combines chemical mutagenesis with exome capture and

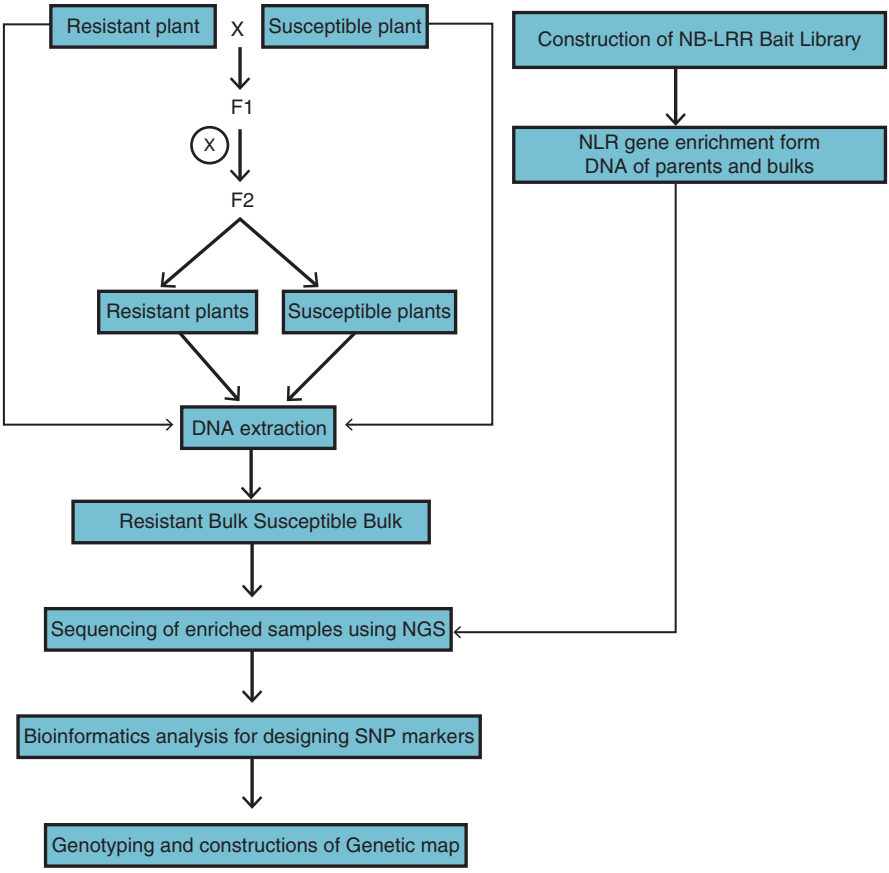

Fig. 6.5 RenSeq methodology used for mapping of disease resistance genes

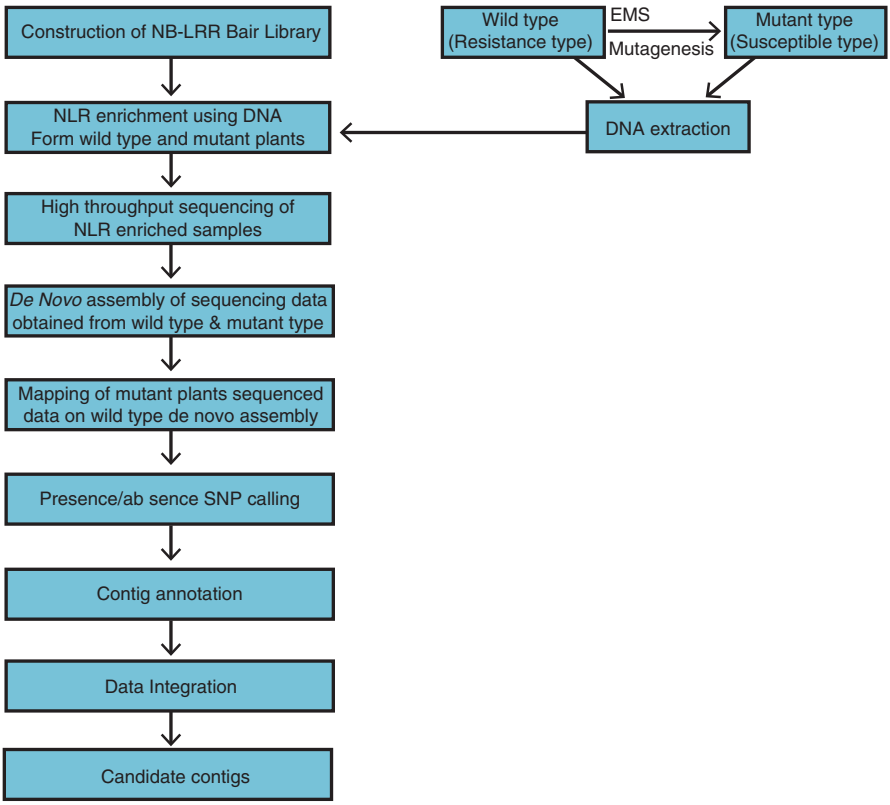

Fig. 6.6 MutRenSeq used for the cloning of disease resistance genes

sequencing for rapid R gene cloning (Fig. 6.6) (Steuernagel et al. 2016). R genes are present in gene families with members in close physical proximity; the genes cannot be cloned via positional cloning especially in plant genomes having large genome sizes that impair cloning due to suppressed recombination. MutRenSeq allows cloning of R genes mediating resistance, which are identified on the basis of mutagenesis through EMS, and loss of resistance mutants with wild-type progenitors. This technique was validated by cloning of previously cloned gene *Sr33* stem rust resistance in wheat, after which two more stem rust resistance genes were isolated *Sr22* and *Sr45* using the same technology. *Sr22* confers resistance to commercially important races of stem rust pathogen, including Ug99 race group which threatened wheat production in Africa. This gene was introgressed to wheat chromosome 7A from the diploid A-genome relatives (*Triticum boeoticum* and *Triticum monococcum*). *Sr45* confers resistance to stem rust pathogen races from Africa, India, and Australia. This gene was introgressed in wheat chromosome 1D from D-genome progenitor *Aegilops tauschii*. Brief methodology involved in MutRenSeq is shown in Fig. 6.2.

6.6.5 *Chromosome Genomics*

Despite of the fact that low-sequencing cost has been offered by next-generation platforms, whole-genome sequencing is still not feasible option for the study of introgressions from wild distant species (species from secondary and tertiary gene pools of wheat) especially for complex plant genomes. Flow cytometry followed by sequencing using NGS is a fascinating new approach that allows the flow sorting of individual chromosomes coupled with sequencing on cost-effective NGS platforms termed as chromosome genomics (Dolezel et al. 2014). IWGSC has also used this approach for the sequencing of individual wheat chromosomes. Genome complexity reduction can be achieved through this technique. Chromosome genomic approach could mark an important step forward for effective utilization of the wild germplasm for wheat improvement. This approach was used by Tiwari et al. (2014) for studying the genomes of distant wild relatives of wheat. They flow sorted $5M^g$ from a wheat/*Aegilops geniculata* disomic substitution line [$DS5M^g$ (5D)] and sequenced it using Illumina HiSeq 2000 system at 50X coverage; the sequences obtained were analyzed for structural and functional annotation. A total of 4236 genes were annotated on $5M^g$. The sorted chromosome was also compared with other species like *Brachypodium distachyon*, rice, sorghum, and barley to find out the synteny blocks. Chromosome $5M^g$-specific SNPs as well as repetitive DNA-based cytogenetic markers were developed and validated. A total of 135 ordered $5M^g$-specific SNPs were located in seven homoeologous deletion bins of chromosome 5D, which provides an excellent resource for screening for $5M^g$-specific introgressions from M-genome species. There were no major chromosomal rearrangements observed between chromosomes $5M^g$ and 5D of wheat. Tiwari et al. (2015) flow sorted the short arm of *Aegilops geniculata* from a wheat line in which it is maintained as a telocentric chromosome. DNA of the sorted arm was amplified and sequenced using an Illumina HiSeq 2000 with ~45x coverage. The sequence data was used for SNP discovery against wheat homoeologous group-5 assemblies. A total of 2,178 unique, $5M^gS$-specific SNPs were discovered, out of which 59 $5M^gS$-specific SNPs were tested by KASPar assay and by Sanger sequencing. Smallest introgression carrying resistance to leaf rust (*Lr57*) and stripe rust (*Yr40*) was identified using selected SNPs.

6.6.5.1 Targeted Chromosome-Based Cloning via Long-Range Assembly (TACCA)

Thind et al. (2017) used chromosome flow sorting followed by sequencing and its long-range assembly to clone *Lr22a* gene. *Lr22a* was introgressed into hexaploid wheat from its wild relative *Aegilops tauschii*. *Lr22a* was previously mapped on chromosome 2D using microsatellite markers. The gene was fine mapped to 0.48cM interval using microsatellite markers by high-resolution mapping. Further to fetch the sequence information of 0.48cM region, chromosome 2D was flow sorted. De novo assembly of chromosome 2D was done using Chicago read pairs which make

use of chromosome contact maps for long-range scaffolding. Chicago sequencing and assembly involve the construction of a Chicago library. Briefly, 200–250 ng of chromosome high-molecular weight DNA (mean fragment length ~100 kb) is reconstituted into chromatin in vitro and fixed with formaldehyde. Fixed chromatin is to be digested with MboI, the 5′ overhangs filled in with biotinylated nucleotides, and then free blunt ends are ligated. After ligation, cross-links are reversed and the DNA was purified from protein. Purified DNA is treated to remove biotin that is not internal to ligated fragments. The DNA is then sheared to ~350 bp mean fragment size, and a sequencing library is generated using restriction enzymes and Illumina-compatible adapters. Biotin-containing fragments are isolated using streptavidin beads before PCR enrichment. The library is then sequenced on an Illumina HiSeq 2500 (rapid run mode) to produce 145 million 150 bp paired-end reads, which provided 30× physical coverage of the chromosome (1–50 kb pairs). An N50 of 9.76 Mb was obtained; both the microsatellite flanking markers were located at a distance of 6.39 Mb in a single scaffold. The scaffold sequence was used to design additional markers by comparing annotated gene sequences to Illumina reads of susceptible parent. The genetic distance was further shortened to 0.09 cM, and physical distance was reduced to 438 Kb which contains nine genes and two pseudogenes. In particular, there was a cluster of two genes encoding nucleotide-binding site-leucine-rich repeat receptor (NLR) and two NLR pseudogenes. *Lr22a* gene was identified using mutagenesis approach and NLR1 corresponded to *Lr22a* gene.

6.6.5.2 Chromosome Flow Sorting

Chromosome flow sorting followed by sequencing has also been extended toward the genome organization and structure of chromosome 5B of wild wheat ancestor *Triticum dicoccoides*, wild emmer wheat the wild relative of *Triticum turgidum*, and the progenitor of durum and bread wheat and maintains a rich allelic diversity among its wild populations. Next-generation sequencing of flow-sorted chromosome 5B of *T. dicoccoides* was done to understand its genomic organization, structure, and function by finding repetitive elements, protein-coding genes, and putative micro-RNAs. Syntenic relationships were also studied with other crop species like *Brachypodium distachyon* (syntenic with *Bd* chromosomes 1 and 4), rice (chromosomes 3, 9, and 12) and *Sorghum bicolor* (chromosomes 1 and 2). Several thousand SNPs were also predicted by mapping of 5B sequenced reads onto the root transcriptomes of two additional *T. dicoccoides* genotypes having contrasting drought tolerance phenotype.

Dissection of chromosomes using flow cytometry has also been done in case of *Aegilops umbellulata* and *Aegilops comosa* and their natural allotetraploid hybrids *Aegilops biuncialis* and *Aegilops geniculata* (Molnar et al. 2011). Chromosome purification of *Aegilops* species (*Aegilops markgrafii*, *Ae. triuncialis*, *Ae. cylindrica*) has been achieved which will provide a great opportunity to study the structure and function of *Aegilops* C genome. It will also facilitate the development of molecular tools to find out the introgression from these species in hexaploid bread wheat (Molnar et al. 2015).

6.7 Genomic Resources to Exploit Wild Germplasm

6.7.1 Genome Sequence of Triticum urartu

Triticum urartu is the diploid wild einkorn wheat, progenitor of the bread wheat A genome. It provides important evolutionary information for bread and durum wheat. It is closely related to einkorn wheat, *T. monococcum*. The genome of *Triticum urartu* accession G1812 was sequenced by the BGI using a whole-genome shotgun strategy on Illumina HiSequation (2000) platform and assembled using SOAPdenovo software. The draft genome assembly was 4.66 Gbp, with a scaffold N50 length of 63.69 kbp. Almost 34,879 protein-coding genes were predicted. The *T. urartu* assembly served as a rich resource for the development of genetic markers for molecular breeding through genomic selection. Almost 739,534 insertion site-based polymorphism (ISBP) markers and 166,309 simple sequence repeats (SSRs) were identified. PCR validation showed that 94.5% of the SSRs and 87% of the ISBP markers gave the expected products and that 33.61% of the SSRs and 10.19% of the ISBP markers were specific to the A genome. Moreover, 28.7% of the SSR loci were polymorphic in bread wheat. Single nucleotide polymorphisms (SNPs) were also identified by re-sequencing of another *T. urartu* accession (DV2138). Around 2,989,540 SNPs were discovered which are useful for the development of SNP markers (Ling et al. 2013). The use of *Triticum urartu* genome sequence has been demonstrated toward the finding of regions where three meta-QTLs (MQTLs_5, 6, and 7) were mapping on chromosome 5A. The available markers for the three meta-QTLs were searched against *T. urartu* scaffold data; ten scaffolds with a total length of 772,014 bp were distributed in the 14-centimorgan (cM) region of MQTL_5; nine scaffolds with a combined length of 783,140 bp were located in the 15 cM region containing MQTL_6; and six scaffolds with an overall length of 529,604 bp were assigned to the 9 cM region harboring MQTL_7. The sequence information of these scaffolds will aid in the development of more polymorphic markers within the three meta-QTL regions and facilitate the identification of their corresponding genes.

6.7.2 Genome Sequence of Wild Emmer Wheat

Wild emmer wheat represents the genome of durum and bread wheat. Though it is too low yielding, it contains characteristics that are being used by plant breeders to improve wheat. Sequencing of wild emmer wheat is under progress, and the study was led by Dr. Assaf Distelfeld of Tel Aviv University's School of Plant Sciences and Food Security and Institute for Cereal Crops Improvement in collaboration with NRGene. The sequence data will help in identifying a number of genes controlling traits selected for domestication by early farmers and that served as foundation for selecting modern wheat varieties. Wild emmer is known as a source of novel variation that can help to improve the nutritional quality of grain as well as tolerance to

diseases and water-limiting conditions. While many modern wheat cultivars are susceptible to water stress, wild emmer has undergone a long evolutionary history under the drought-prone Mediterranean climate. Thus, utilization of the wild genes in wheat breeding programs promotes producing more yield for less water.

6.7.3 *Genome Sequence of Durum Wheat*

Durum wheat is the tenth most important crop worldwide. It is likely to be the first domesticated crops in the Fertile Crescent. Durum is tetraploid wheat with a genome of about 12 Gb, domesticated from wild emmer in the Fertile Crescent about 10,000 years ago. The International Wheat Genome Sequencing Consortium (IWGSC) did the sequencing of cultivated durum wheat cultivar "Svevo" and generated high-quality reference. Sequencing was done with Illumina short paired-end and long mate pair protocols up to 270× coverage, and reads were assembled with the NR-Gene DeNovoMAGIC™ pipeline. Genome was assembled to 10.5 Gb of sequence with N50 and L50 of 493 Mb and 6 Mb. The assembled scaffolds have been anchored to saturated genetic maps based on Svevo x Zavitan RIL population; 95% of the scaffolds have been anchored and 90% of them are oriented.

6.7.4 *Genome Sequence of Synthetic Wheats*

Chapman et al. (2015) sequenced two (hexaploid) bread wheat lines, "Synthetic W7984" and "Opata M85," along with a set of 90 doubled haploid (DH) lines derived from W7984/Opata F_1 hybrids, the "SynOpDH" population (Sorells et al. 2011). A total of 175-fold coverage had been generated based on Illumina whole-genome sequence. De novo sequence assembly represented 9.1 Gbp of 17 Gbp genome of polyploid wheat with a N50 of 6.7 Kb, and 7.1 Gbp was assigned to different chromosomal locations. The assembled genome was validated using SNP map already available in "SynOpDH" population. Also ultrahigh-density linkage map was constructed using POPSEQ approach by whole-genome sequencing of 90 DH individuals obtained from the cross between Synthetic W7984 and Opata M85.

6.7.5 *Genome Sequence of* Triticum monococcum

The diploid species *Triticum monococcum* ($2n=2x=14$, A^mA^m), commonly known as einkorn wheat, was widely cultivated in Fertile Crescent. It was domesticated from its wild progenitor *T. boeoticum. T. monococcum* provide an ideal cereal model to study diversity of important traits and genetic diversity after domestication and also found to be suitable for wheat genetic improvement. The A^m genome of *T. monococcum* and the A^u genome of *T. urartu* are closely related and diverged 0.5–1 million

years ago (Huang et al. 2002; Dvorak and Akhunov 2005). The genome sequence of *Triticum monococcum* has been sequenced by IWGSC and data is available on its website. Genome-wide transcriptomes of two *Triticum monococcum* subspecies, the wild winter wheat *T. monococcum* ssp. *aegilopoides* (accession G3116) and the domesticated spring wheat *T. monococcum* ssp. *monococcum* (accession DV92) were studied by Fox et al. (2014) by generating de novo assemblies of RNA-Seq data derived from both etiolated and green seedlings. The de novo transcriptome assemblies of DV92 and G3116 represent 120,911 and 117,969 transcripts, respectively. They were mapped to the bread wheat, barley, and *Triticum urartu* genomes. Bacterial artificial chromosome (BAC) library had also been constructed from *T. monococcum* (Lijavetzky et al. 1999) which was used as a source to find 450 Kb physical contig spanning *Lr10* resistance locus in hexaploid wheat (*Triticum aestivum*) (Stein et al. 2000). Chanteret et al. (2004) obtained sequence of the 101 Kb BAC of *Triticum monococcum* containing hardness locus showing good microcolinearity with rice.

6.7.6 *Transcriptome Sequence of* Aegilops sharonensis

Aegilops sharonensis Eig (Sharon goat grass) is a wild diploid relative of wheat within the *Sitopsis* section of *Aegilops*. This species represents an untapped reservoir of genetic diversity for traits of agronomic importance, especially as a source of novel disease resistance (Yu et al. 2017). Sequencing of the cDNA from leaf tissue of two geographically distinct *Aegilops sharonensis* accessions (1644 and 2232) was done using the 454 Life Sciences platform. About 62,243 nonredundant sequences were found on doing reciprocal blast hits (BRBH) against 6 previously characterized grass proteomes and identified 139 belonging to plant disease resistance genes of the nucleotide-binding site-leucine-rich repeat class. Based on the nonredundant sequences, 37,743 single nucleotide polymorphisms (SNP) were predicted, equivalent to one per 1,142 bp. The *Aegilops sharonensis* leaf transcriptome provides a rich source of sequence and SNPs for this wild wheat relative. These sequences can be used with existing monocot genome sequences and EST sequence collections (e.g., barley, *Brachypodium*, wheat, rice, maize, and *Sorghum*) to assist with genetic and physical mapping and candidate gene identification in *Aegilops sharonensis*. These resources provide an initial framework to further build on and characterize the genetic and genomic structure of *Aegilops sharonensis* (Bouyioukos et al. 2013).

6.7.7 *Transcriptome Sequence of* Agropyron cristatum

Agropyron cristatum is a wild grass of the tribe Triticeae that is widely grown in harsh environments. *It* carries many resistance genes that could be used to broaden the genetic diversity of wheat. Transcriptome sequencing of the flag leaf and young

spike tissues of a representative tetraploid *Agropyron cristatum was done by* Zhang et al. (2015). More than 90 million reads from the two tissues were assembled into 73,664 unigenes. The extent of specific genes and rare alleles makes *Agropyron cristatum* a vital genetic reservoir for the improvement of wheat. Altogether, the available gene resources in *Agropyron cristatum* facilitate efforts to harness the genetic diversity of wild relatives to enhance wheat.

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Chapter 7
Genetics and Applied Genomics of Quality Protein Maize for Food and Nutritional Security

P. K. Agrawal, M. G. Mallikarjuna, and H. S. Gupta

Abstract Maize (*Zea mays* L.) is an important food and feed crop of the world. Together with rice and wheat, it provides around 40% of the food calories to more than 4.5 billion people in 94 developing countries. It also provides nearly 50% of the dietary protein for humans. In Africa and some of the Asian countries, almost 90% of maize grown is for human consumption and may account for 80–90% of the energy intake. In India, it is the third most important food crop after rice and wheat, both in terms of area and production. India is the fifth largest producer of maize in the world contributing 3% of the total global production. Protein malnutrition is widespread in the developing and underdeveloped countries, where 780 million people are affected by the same. Maize is the leading cereal in terms of production and accounts for 15% of proteins and 20% of calories requirement of the world. Protein malnutrition is caused by lack of access to adequate quantity and better quality protein intake and usually affects children and elderly persons. Maize, however, lacks adequate amounts of the essential amino acids, namely, lysine and tryptophan. Decades of efforts by maize researchers lead to the development of nutritionally superior maize cultivar popularly called as quality protein maize (QPM), which has twice the amount of lysine and tryptophan, thus making its quality as good as casein of milk. The *o*2 allele along with modifiers for tryptophan and lysine content and grain hardness made QPM agronomically suitable for cultivations. Intensive efforts were made by many workers to understand the genetics, molecular mechanism of QPM modifiers and applied these genomics knowledge to developed MAS-based QPM inbreds and commercial hybrids. All those studies and concerted efforts led to development and utilization of QPM. The area under QPM globally is more than 9.0 million hectares. Several reports were available on positive impact of QPM on children and adults. It has also been demonstrated in poultry and

P. K. Agrawal (✉)
National Agricultural Science Fund, Indian Council of Agricultural Research, New Delhi, India

M. G. Mallikarjuna
Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India

H. S. Gupta
ICAR-Indian Agricultural Research Institute, New Delhi, India

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piggery, resulting in increased egg production and egg quality parameters and body mass. The area under QPM and consumption of QPM can be increased significantly by providing policy supports for QPM.

Keywords Lysine · Malnutrition · MAS · Maize · Nutrition · Policy · Protein · QPM

7.1 Introduction

Tremendous advances in agricultural sciences have although helped in enhancing food production remarkably; yet, malnutrition continues to be a worldwide problem especially in the developing and underdeveloped countries. Globally, 795 million people are chronically undernourished, and, out of this, nearly 780 million live in developing world (https://www.worldhunger.org). The problem is more severe in preschool children and women. In developing countries, about 32% of preschool children are stunted, and 20% are underweight due to protein malnutrition alone (Black et al. 2008). Cereals are the major contributors to global food security with an annual production of ~2.3 billion tonnes. Out of it, nearly one billion tonnes is used for human consumption, 750 million tonnes for animal feed, and the remaining 500 million tonnes for industrial use as well as for seeds and other purposes (FAO 2015; Shewry 2007). Thus, cereals contribute more than 50% of the dietary protein requirement globally and 70% in developing countries (Gibbon and Larkins 2005). Among cereals, maize, wheat, and rice contribute over 85% of total cereal production and accounts 42.5% of world calorie requirement (Shiferaw et al. 2011; FAO 2015). However, in most of the developing nations where single multi-cereal-based diet predominates, the nutritional profile of such cereal(s) assume great significance.

Maize leads the global cereal production with an annual output of 1060 million tonnes and contributes more than 100 million tonnes of proteins (assuming 10% protein) during the year 2016–2017 (http://www.fao.org/faostat/). It is the major source of daily diet in Sub-Saharan Africa, Latin America, and Asia. Although, normal maize is a good source of basic dietary requirements, it cannot provide all the essential amino acids, which are not manufactured in human body (Nelson 1969; Gupta et al. 2013). Approximately 70% of the maize protein is composed of alcohol-soluble prolamins known as zeins, namely, α, β, γ, and δ (Gupta et al. 2009). These protein fractions are rich in glutamine, leucine, and proline and distributed in a distinctive pattern in grain (Gibbon and Larkins 2005). Zeins in normal maize possess unbalanced amino acid composition with reduced concentration of essential amino acids, namely, lysine and tryptophan (Gupta et al. 2009). Reduced quantity of essential amino acids in zeins of normal maize protein brings down the biological value of maize protein to 40% of milk protein. This makes maize a poor source of protein. Supplementation of protein sources from legumes and animal products is often not viable because of cost factor. Several natural mutants with higher lysine and tryptophan, namely, *opaque-2* (*o2*), *floury*-2 (*fl2*), *opaque*-7 (*o7*), *opaque*-6 (*o6*), and *floury-3* (*fl3*), were identified in maize (Ignjatovic-Micic et al. 2008).

Among these mutants, *o2* mutant was found to be most amenable for genetic manipulation of lysine and tryptophan owing to its ability to accumulate higher lysine and tryptophan content (Bressani 1992) without reducing or altering the total protein concentration. Further, the increased concentration of lysine and tryptophan in *o2* mutants increased the biological value of maize protein to 90% of the milk protein (Bressani 1992). However, the desirable nutritive value of *o2* mutants was found to be associated with undesirable agronomic traits such as dull and chalky grains and lower grain weight and susceptibility to several diseases and insect pests. Painstaking efforts at CIMMYT, Mexico, by Vasal and Villegas led to breaking of undesirable linkage between the nutritive value and agronomically undesirable characters. Further, *o2* in combination with modifiers lead to the development of quality protein maize (QPM) (Vasal 2000, 2001, 2002). The QPM, thus, overcame all the defects associated with *o2*: soft to harder endosperm, higher yield potential, and resistance to pests and diseases. The nutritional impact of quality protein maize was well demonstrated by several international programs in Africa and Latin American countries (https://www.povertyactionlab.org).

7.2 Maize Endosperm and Its Mutants

A typical maize kernel is characterized by an endosperm and embryo. The endosperm is surrounded by an outermost aleuron layer; the starchy endosperm cells and protein are tightly packed just beneath the aleuron layer. Packing density of endosperm cells gives a typical vitreous (glassy) or starchy appearance to maize kernel. Generally, the endosperm is composed of around 90% of starch and 10% protein. The maize endosperm proteins can be classified into four types: (i) water-soluble albumins (3%), (ii) salt-soluble globulins (3%), (iii) alkali-soluble glutelins (34%), and (iv) alcohol-soluble prolamins (zeins) (60–70%) (Vasal 2000; Gibbon and Larkins 2005). Further, zeins are specific to maize endosperm (Prasanna et al. 2001). The zeins are characterized into one major class called α-zeins (19 and 22 kDa) and three minor classes, namely, β- (15 kDa), γ- (16, 27, and 50 kDa), and δ-zeins (10 and 18 kDa), and are encoded by different classes of structural genes (Coleman and Larkins 1999). The zeins are known to be very poor in lysine content (0.01%) compared to higher concentration (>2 g/100 g protein) in case of albumins, globulins, and glutelins. On the contrary, embryo protein is dominated by the albumin fractions (>60%) which are superior in terms of amino acid composition and nutritional quality (Vasal 2000). The presence of higher amount of zeins with negligible concentration of lysine in maize endosperm imparts negative effects on growth of animals (Osborne and Mendel 1914). In order to enhance the concentration of lysine in zein proteins, several high-lysine mutants were identified and characterized to overcome the limitations of zeins. *Opaque* mutants with 50% reduced zein fraction and increased concentration of nutritionally superior non-zein endosperm proteins were identified and successfully employed in the breeding program (Gupta et al. 2013; Babu and Prasanna 2014).

7.2.1 Genetics of Endosperm Mutants

A large number of maize endosperm mutants, causing modification of the endosperm and its constituents, have been reported (Thompson and Larkin 1994; Hunter et al. 2002). All the opaque mutants, namely, *o1* (Emerson et al. 1935), *o2* (Mertz et al. 1964), *o5* (Gibbon and Larkins 2005), *o6* (Ma and Nelson 1975), *o7* (Misra et al. 1972; Burr and Burr 1982), *o9–11* (Nelson 1981), and *o13–17* (Dannenhoffer et al. 1995; Gibbon and Larkins 2005; Yang et al. 2005) were recessive in nature, whereas the floury mutants, namely, *fl1* (Emerson et al. 1935), *fl2* (Nelson et al. 1965), and *fl3* (Ma and Nelson 1975) are semidominant in nature. The mutants, mucronate (*Mc*) (Salamini et al. 1983) and *Defective endosperm B30* (*De-B30*) (Salamini et al. 1979), showed dominant inheritance. In addition to simple inheritance, some of the genes show epistatic interactions and dosage effects. The *o2* and *o7* genes were reported to be epistatic over *fl2*, whereas synergetic effects were observed between *o2* and *Mc* and *o2* and *o16* (Prasanna and Sarkar 1991; Zhang et al. 2010, 2013). The dosage effects were reported for mutations *De-B30*, *fl2*, and *fl3*, where the kernel opacity and protein quality depend on the dosage of the recessive alleles in the triploid endosperm (Soave et al. 1981; Vasal 2002).

Furthermore, spontaneous mutant *o7* isolated from W22 was found located on chromosome 10 (Tsai and Dalby 1974). The *o7* possess starchy endosperm at maturity, reduced endosperm weight, and total protein content (Di Fonzo et al. 1979). Like other opaque mutants, *o*7 also inhibits the synthesis of all zein classes of proteins (Misra et al. 1972; Lee et al. 1976; Hartings et al. 2011). A new endosperm mutant *o16* was identified by Yang et al. (2005) and positioned on long arm of chromosome 8. The *o16* showed increased lysine content (~0.36%) and was found to be linked to the molecular marker umc1141. Unlike other opaque mutants, endosperm phenotype of *o5* mutant is caused by reduction of galactolipids of the endosperm without any alteration in the zeins content (Myers et al. 2011). On the other hand, floury mutants share many of the common features of opaque mutants such as reduced zein fraction, soft chalky endosperm texture, and inability to accumulate sufficient dry matter (Vasal 2002). The *fl2* mutation is caused by the accumulation of the improperly processed of 24 kDa α-zein precursor protein, and the resultant protein bodies are not only smaller than normal, but they are also asymmetrical and misshapen (Coleman et al. 1997). However, the *fl1* mutation changes the location of 22 kDa α-zeins within the protein body and does not affect the amount and composition of zeins per se (Holding et al. 2010). The other group of mutants such as *fl4*, *De-B30*, and *Mc* are associated with improper distribution of zeins and protein body deformation like *fl2* mutation (Coleman et al. 1995; Kim et al. 2006; Wang et al. 2011, 2014).

Although endosperm mutant *o2* was reported as early as in the 1920s, the nutritional significance was first showed by Mertz and co-workers in the 1960s (Singleton 1939; Mertz et al. 1964; Nelson et al. 1965). Thereafter, it has extensively been used and incorporated in a large number of maize lines; however, due to pleiotropic effect on the endosperm in terms of kernel softness, disease susceptibility, short storage time, etc., it could not become popular.

7.2.2 *Molecular Mechanism Underlying Endosperm Modifications*

7.2.2.1 Opaque Mutants

The discovery of *o2* mutant of maize in 1960s aroused great interest as it helped in enhancing biological value of maize protein by increasing lysine and tryptophan-the two essential amino acids that are not synthesized in human as well as livestocks. The *o2* mutant was most extensively studied owing to its widespread usage in the successful development of global QPM development programs. The wild allele *O2* codes for defective basic domain-leucine zipper transcription factor and regulates expression of the 22 kDa α-zeins and other endosperm-specific genes (Schmidt et al. 1987, 1990; Damerval and De Vienne 1993; Habben et al. 1993). The defective allele *o2* represses the expression of 22 kDa α-zeins genes and thereby reduces the production of zeins fraction (Schmidt et al. 1987, 1990). Unlike *o2*, the other mutations *fl2*, *fl4*, and *De-B30* encode for defective signal peptides and result in disrupted zein deposition. In *fl2* the point mutation (alanine, valine) at 22 kD α-zein signal peptide affects the processing and removal of signal peptide from mature protein. Similarly, *fl4* and *De-B30* are resulted from mutations that cause the signal peptides to remain attached with the 19 kD α-zein, resulting in aggregation of these proteins in the endoplasmic reticulum (Gillikin et al. 1997; Wang et al. 2014). In contrast to mutations at signal peptides, *Mc* gene results from a 38 bp deletion and leads to a frameshift mutation in the 16 kD γ-zein (Gibbon and Larkins 2005; Kim et al. 2006).

7.2.2.2 Endosperm Modifiers

Considerable information is available on molecular and biochemical basis of kernel texture modification in QPM. Proteomics of QPM lines showed increased granule-bound starch synthase I in the soluble non-zein fraction and resulted in shorter amylopectin branches and increased starch grain swelling (Gibbon et al. 2003). *o2* modifiers are semidominant genes and express in both *o2* and normal genetic backgrounds; however, higher expression rate during seed development was observed in modified opaque lines. The increased modifier gene expression appears to be a consequence of enhanced mRNA transcription or transcript stability (Geetha et al. 1991). The increased expression rate of *γ-zein A* plays important role in kernel opaqueness. The expression rate of γ-zein A/γ-zein B after 18 days of pollination in modified opaque-2 was 40:1 as compared to 1:1 in wild-type and 3:1 in *o2* genotypes (Burnett and Larkins 1999). In addition to *o2*, prolamine-box binding factor (PBF) regulates zein and starch synthesis. The *o2* and *PbfRNAi* showed reduced starch synthesis ~5% and ~11%, respectively. Whereas, double-mutant *PbfRNAi-o2* showed reduced starch synthesis by 25%. Transcriptome assay revealed that the expression >1000 genes related to sugar and protein metabolism was affected in *PbfRNAi*, *o2*, and *PbfRNAi-o2* mutants (Zhang et al. 2016).

7.3 Development of Quality Protein Maize (QPM)

The untiring efforts by Vasal and co-workers at CIMMYT during the 1970s and 1980s helped in overcoming the major drawbacks of *o2* mutants, namely, soft endosperm, shorter shelf life, disease susceptibility, and low yield, when high-lysine mutants *o2* was combined with modifiers. Whereas the one set of modifier genes helped in overcoming softness of the endosperm and gave rise in vitreous and hard endosperm, the other set of modifiers helped in increasing lysine and tryptophan content (Tandzi et al. 2017). Thus, the unique combination of *o2* and endosperm hardness led to the development of quality protein maize (QPM) (Vasal et al. 1980). The discovery of endosperm modifiers or *opaque modifiers* (*Opm*) revolutionized QPM breeding (Villegas et al. 1992). The endosperm modifiers possess multiple loci and complex phenotypic effects (Vasal et al. 1980; Belousov 1987; Geevers and Lake 1992). Genetic analysis of endosperm modifiers identified several quantitative trait loci (QTLs) in various source of populations. QTLs associated with *o2* endosperm modification in the QPM backgrounds of Pool 33 (CIMMYT in Mexico; Vasal et al. 1980; Bjarnason and Vasal 1992) and K0326Y (South Africa; Geevers and Lake 1992) were mapped. Pool 33 and K0326Y were crossed with a starchy endosperm Midwestern inbred W64Ao2. QTLs for *Opms* were identified in F_2-F_3 progenies segregating for a vitreous or starchy kernel phenotype by bulked segregant analysis. Three *Opms* QTLs for Pool 33 (bins, 6.03, 6.04, and 7.02) and three QTLs for K0326Y (bins, 7.02, 9.03, and 9.05) were identified. Additional seven QTLs explaining 75% of phenotypic variation were detected in the F_2 population (K0326Y QPM x W64Ao2). The bins 7.02 and 9.04/9.05 F_2 corresponded with the QTLs identified by BSA explained 40% of the phenotypic variation (Holding et al. 2008). The validation and characterization of identified QTLs for *Opms* were carried by Holding and co-workers in 2011. Genetic linkage analysis of the RIL (K0326Y QPM × W64Ao2) population validated the three of the previously identified QTLs associated with *o2* endosperm modification. The QTL located on chromosome 7 (umc1036-γ-zein) explained maximum phenotypic variation for vitreousness (38%), breakage mean (22.7%), and density (37.8%) (Holding et al. 2011). Five QTLs falling on chromosome number 5, 7, and 9 and explained 38.6% of the total phenotypic variance for *Opms* were identified in the $F_{2:3}$ population developed from hill-adopted Indian maize genotypes (VQL2 × VQL8) (Babu et al. 2015). The identified major QTLs at bin 7.02, which are candidates for *Opm* genes, showed increased 27 kDa γ-zein gene expression (Holding et al. 2008). However, the QTLs linked to *o15* showed decreased 27 kDa γ-zein expression (Dannenhoffer et al. 1995). The γ-RNAi knockdown of 27 and 16 kDa γ-zeins showed only partial opacity by an incomplete embedding of starch granules in the vitreous area (Wu et al. 2010).

Although several QTLs were identified and co-located candidate genes were characterized, the molecular mechanism by which *Opm* creates vitreous phenotype is not understood clearly. Biochemical characterization of QPM genotypes revealed

the association between large amount of the cysteine-rich 27 kDa γ-zein storage protein and dosage of *Opm* genes (Ortega and Bates 1983; Wallace et al. 1990; Geetha et al. 1991; Lopes and Larkins 1991). Furthermore, there is a correlation between γ-zein content and the number of protein bodies in the endosperm, as verified by the *o15* mutant, which has a reduced level of γ-zein and contains half of the normal number of protein bodies. γ-zein ability to form covalent linkage with other cysteine-rich proteins that leads to the formation of a tightly linked proteinaceous network surrounding the starch grains could be a reason for its association with a number of protein bodies (Dannenhoffer et al. 1995). Yuan et al. (2014) demonstrated that deletions in γ-zein caused intermediate 27 and 50 kDa γ-zein levels and were semivitreous owing to indicating haplo-insufficiency of these gene products in *o2* endosperm modification. The γ-zein as an *o2* modifier gene within the largest QPM quantitative trait locus may suggest the 50 kD γ-zein also contributes to endosperm modification. Recently, Liu et al. (2016) identified a quantitative trait locus (qγ27) affecting the expression of 27 kDa γ-zein in the same region as the major *o2* modifier loci on chromosome 7. The qγ27 resulted from a 15.26-kb duplication at the 27 kDa γ-zein locus. Although duplication might have occurred before maize domestication, the gene structure of qγ27 appears to be unstable, and the DNA rearrangement frequently occurs at this locus owing to enhanced response for artificial selection.

7.4 Breeding QPM Varieties and Hybrids

7.4.1 Conventional Breeding

The development of composites and hybrid cultivars is the major targets in cross-pollinated crops like maize. The synthesis of QPM hybrids necessitates production of inbred lines with higher kernel lysine and tryptophan content, which could be achieved either through development of entirely new inbred lines or conversion of agronomically elite maize inbred lines through introgression breeding. Initially, conventional breeding was directed toward development of reliable new inbreds with higher kernel lysine content. Owing to higher protein quantity and quality of maize kernel germ/embryo, efforts were made to increase the germ-to-endosperm ratio and selection for multiple aleuron layers. However, the germ-to-endosperm ratio had a negative association with the shelf life of maize. Additionally, many of these efforts were based on recurrent selection. The population-based breeding approaches for enhancement of lysine content through alteration of kernel phenotype were not much successful owing to narrow genetic base and high demand for resources and laboratory facilities (Babu and Prasanna 2014).

The discovery of nutritional importance of *opaque* mutants leads to accelerated breeding efforts for the development of high-lysine-rich maize. High-lysine *opaque* mutants provided avenues to exploit the double *o2* mutants simultaneously in com-

bination with endosperm and amino acid modifiers. The double *o2* mutants in combination with modifiers lead to development of high-lysine maize with vitreous kernel (Vasal 2001). Analysis of endosperm modification of maize kernels in segregating generations was started in 1969 by Lonnquist and Asani (Babu and Prasanna 2014). The initial approaches for development of QPM stocks were based on population improvement strategies, namely, intrapopulation selection for kernel modification *o2* background and recombination of superior vitreous *o2* families. These strategies were employed in large-scale development of QPM donor stocks in temperate, tropical, and sub-tropical germplasm (Vasal 2001). More recently, novel conventional breeding approaches such as "modified backcross-cum-recurrent selection" and "pedigree backcrossing scheme" were employed for rapid and efficient conversion of inbred lines for *o2* and kernel hardiness (Vasal 2001). Presently, pedigree and backcross breeding with QPM × QPM- and QPM × non-QPM-based crosses are used for the development and conversion of QPM inbred lines. Efforts to develop QPM hybrids at CIMMYT were initiated during 1985 owing to advantage of hybrids as compared to OPV-QPM. In addition to higher yield, stable and uniform endosperm modification, and seed purity, QPM hybrids also ensured minimal efforts to monitor the protein quality owing to their genetic uniformity (Vivek et al. 2008). Several QPM heterotic combinations were tested in national and international breeding programs, and some of them were released for regular cultivation. The list of QPM cultivars developed through conventional breeding are collated in Table 7.1.

The promising QPM composites and hybrids developed at CIMMYT were introduced in India, and after testing in All India Coordinated Project on maize, they were released for commercial cultivation. Apart from this, several QPM hybrids were developed in Africa as well as in India from the parental lines supplied by SK Vasal. Efforts were also made to improve the QPM hybrids for drought stress. The screening of QPM genotypes for drought stress identified several germplasms as source for drought tolerance breeding and early maturity (Zaidi et al. 2008; Pfunde and Mutengwa 2016). The inbred CML18 showed early maturity under drought stress (Pfunde and Mutengwa 2016). The heterotic combinations of QPM inbreds, namely, CML3 × CML13 and CML5 × CML9, gave better grain yield (~3 t/ha) under drought stress (Pfunde and Mutengwa 2016). Breeding program for grain yield and tolerance against other stresses is also being carried out by several research groups.

7.4.2 Molecular Marker–Assisted Breeding (MAB)

Although conventional breeding strategies have been used to develop a large number of QPM stocks and convert commercial lines to QPM forms, the procedures are tedious and time consuming. In order to overcome breeding difficulties associated with recessive nature of *o2* and modifiers such as large number of generations during backcross program, rigorous biochemical testing after every generation, selection for multiple endosperm modifiers, and demand for huge resources lead to look for an alternative approach. Advances in genomics science and technology

Table 7.1 List of some of the QPM cultivars developed through conventional breeding

S. No.	Cultivar	Type of cross	Pedigree	Endosperm	Year	Country	Grain
1	AMH760Q (Webi)	Three-way	–	Hard	2010	Ethiopia	White
2	AMH852Q	Three-way	–	Hard	2016	Ethiopia	White
3	BHQP542 (Gabissa)	Three-way	(CML144 × CML159) × CML 176	Hard	2001	Ethiopia	White
4	BHQPY545 (Kello)	Single	CML161 × CML165	Hard	2008	Ethiopia	Yellow
5	BHQPY548	Three-way	–	Hard	2015	Ethiopia	White
6	GH-132-28	Single	P62 × P63	Hard	1997	Ghana	–
7	HB- PROTICTA	Three-way	(CML144 × CML159) × CML176	Hard	–	Guatemala	White
8	HQ INTA- 993	Three-way	(CML144 × CML159) × CML176	Hard	–	Nicaragua	White
9	HQ-31	Three-way	(CML144 × CML159) × CML176	Hard	–	Honduras	White
10	HQ-61	Three-way	(CML144 × CML159) × CML176	Hard	1999	El Salvador	White
11	HQPM-1	Single	HKI193-1 × HKI163	Hard	2005	India	Yellow
12	HQPM-4	Single	HKI-193-2 × HKI 161	Hard	2010	India	Yellow
13	HQPM-5	Single	HKI163 × HKI 161	Hard	2007	India	Yellow
14	HQPM-7	Single	HKI193-1 × HKI161	Hard	2008	India	Yellow
15	ICA	Three-way	(CML144 × CML159) × CML176	Hard	–	Colombia	White
16	KH500Q	Three-way	(CML 144 × CML 59) ×CML181	Hard	–	Kenya	White
17	KH631Q	Three-way	(CML 144 × CML 159) × CML 182	Hard	–	Kenya	White
18	Lishe-H1	Three-way	(CML144 × CML159) × CML176	Hard	–	Tanzania	White
19	Lishe-H2	Top	Obatampa × (CML144 × CML159)	Hard	–	Tanzania	White
20	Melkassa 1Q	OPV	–	–	2013	Ethiopia	Yellow
21	Melkassa 6Q	OPV	–	–	2008	Ethiopia	White
22	MHQ138	Three-way	–	Hard	2012	Ethiopia	White
23	Pratap QPM Hybrid-1	Single	DMRQPM-106 × HKI-193-1	Hard	2013	India	Yellow

(continued)

Table 7.1 (continued)

S. No.	Cultivar	Type of cross	Pedigree	Endosperm	Year	Country	Grain
24	Protina	Composite	(Jowatigua x Antigua car II) o2 x (Doeto × G.C.C.) o2	Soft	1971	India	Yellow
25	Quian 2609	Single	Tai 19/02 × CML171	Hard	–	China	Yellow
26	Rattan	Composite	J1o2	Soft	1971	India	Yellow
27	Shakti	Composite	JLo2, Cuba1Jo2, Antigua 2Do2	Soft	1971	India	Yellow
28	Shakti-1	Composite	Antigua, Ver 181 HEo2, Amarillo crstallino HEo2, Ant Rep Dom, HEo2, temperate HEo2	Hard	1997	India	Yellow
29	Shaktiman-1	Three-way	(CML142 x CML150) x CML186	Hard	2001	India	White
30	Shaktiman-2	Single	CML176 × CML186	Hard	2004	India	White
31	Shaktiman-3	Single	CML161 × CML163	Hard	2006	India	Yellow
32	Shaktiman-4	Single	CML161 × CML169	Hard	2006	India	Yellow
33	Shaktiman-5	Single	CML161 × CML165	Hard	2013	India	Yellow
34	Zhong Dan 206	Single	–	Hard	1988	China	–
35	Zhong Dan 9409	Single	Pool 33 × Temp. QPM	Hard	–	China	Yellow
36	ZS261Q (CZH01021)	Double	(CZL01006 × CML176) × (CZL010 05 × CML181)	Hard	–	Zimbabwe	White

facilitated the adoption of marker-assisted selection (MAS) for rapid and efficient conversion of commercial cultivars into QPM version (Ribaut and Hoisington 1998; Xu and Crouch 2008; Gupta et al. 2009). MAS use molecular markers to select or deselect the target genomic region (*o2*) involved in expression of desired trait without or mere disturbance of the background genomic region. Foreground selection (using gene-based/gene-linked markers) of MAS helps to retain *o2* allele in segregating generations and background selection (using markers polymorphic between the donor and recurrent parents) that aid in recovering individuals with desired genotype at the target locus. Cloning and molecular characterization of *o2* gene identified three *o2*-based SSR markers, namely, phi057, phi112 and umc1066 (Lin et al. 1997; Bantte and Prasanna 2003; Yang et al. 2008). The identified SSRs offered remarkable advantage in foreground selection. "Foreground selection" for the *o2* allele using gene-based SSR markers and "background selection" (using markers polymorphic between the donor and recurrent parents) aid in recovering individuals with desired genotype at the target locus, besides high levels of recovery of recurrent parent genome, within two backcross generations. Babu et al. (2005) and Gupta et al. (2009) developed MAS-based breeding pipeline for rapid conversion of commercial non-QPM inbreds to QPM version within two generation of backcrosses and phenotypic selection for kernel modification and agronomical and biochemical traits in two subsequent selfed generations (Fig. 7.1). However, it was experienced by our team at ICAR-VPKAS, Almora that the number of markers for background selection needs to be large (preferably >100) for a viable MAS-based breeding approach. This strategy allows selection and fixing of large segregating generation for the *o2* in segregating generations with simultaneous reduction of linkage drag by using flanking markers for recipient allele types. This strategy can thus be implemented in a cost- and time-effective manner as compared to phenotypic selection alone (Dreher et al. 2003; Babu and Prasanna 2014). The flow chart in Fig. 7.1 shows the schematic diagram for the same.

Availability of simple, straightforward MAS strategy and low cost of genotyping enabled the plant breeders to adopt MAS as a method of choice for rapid development of QPM version for the development of commercial cultivars. Additionally, it also facilitated the diversification of genetic base of QPM cultivars to suit the need of targeted agroecologies. There are very good number of successful examples of MAS for the development of QPM hybrids available in Indian maize breeding programs (Table 7.2) (Babu et al. 2005; Gupta et al. 2009, 2013; Prasanna et al. 2010). Using the method, many normal maize inbreds and hybrids have been converted into QPM versions and were released for commercial cultivation (Babu et al. 2005; Gupta et al. 2009). Five successful examples of developing QPM hybrids that were converted through MAS are presented below.

Vivek QPM 9 Gupta et al. (2009) converted successfully a promising maize hybrid, namely, Vivek Maize Hybrid 9, to QPM version using MAS. Vivek QPM 9, the resulting QPM hybrid, matures in 85–90 days and yields up to 6.0 t/ha. The performance of Vivek QPM 9 (5.8 t ha^{-1} in zone I and 5.4 t ha^{-1} in zone IV) was at par with Vivek Maize Hybrid 9 (5.9 t ha^{-1} and 5.4 t ha^{-1} in zone IV) in both the zones over years. It

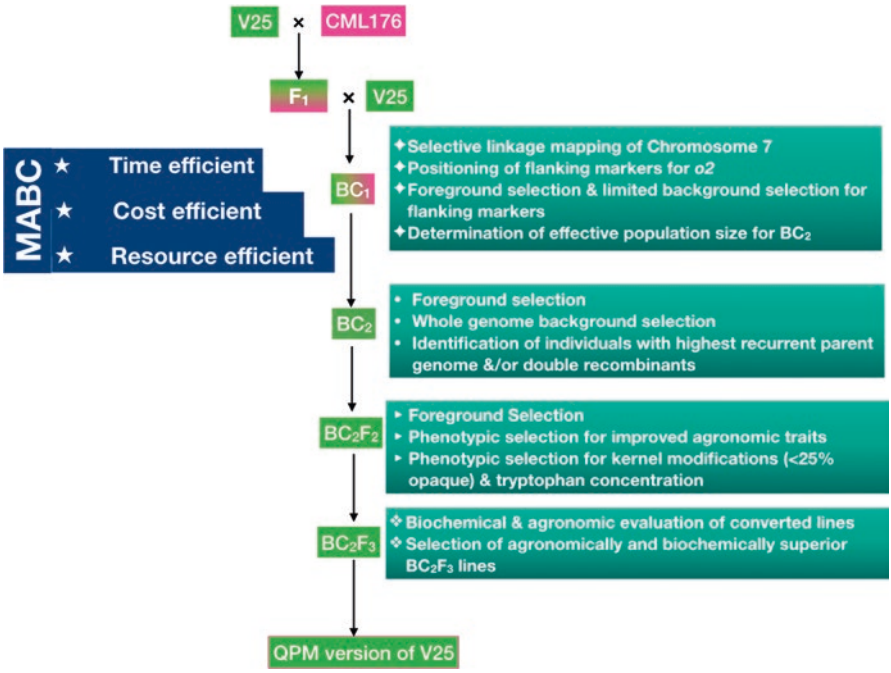

Fig. 7.1 Schematic diagram for converting a normal maize inbred (V25) to QPM version through MAS. (Modified from Babu et al. 2005)

also showed 41% higher tryptophan in the endosperm as compared to Vivek Maize Hybrid 9. It possesses equal level of resistance to *turcicum* blight – the most important disease of maize crop in the hills. Based on the performance for 2 years in zone 1 and zone IV, Vivek QPM 9 was released in 2008 for commercial cultivation in zone I and zone IV in India and for the organic conditions in the hills of Uttarakhand (Fig. 7.2).

Vivek QPM 21 Vivek QPM 21 (QPM version of Vivek Maize Hybrid 21) shows >70% enhancement in tryptophan over the original hybrid, Vivek Maize Hybrid 21. The tryptophan content of Vivek QPM 21 is 0.85, whereas it is 0.49 for Vivek Maize Hybrid 21. Vivek QPM 21 was also tested in the All India Coordinated Trial of *Kharif* 2007, 2008, and 2009, in which it performed equally well in respect to grain yield and other agronomic traits over non-QPM national check, Vivek Maize Hybrid 17 and Vivek Maize Hybrid 21. Vivek Maize Hybrid 21 was released for commercial cultivation in zone I, II, and IV in 2006. The parents of this hybrid were converted into the QPM version using DNA markers, and this hybrid was reconstituted by crossing VQL 1 and VQL 17. This QPM hybrid shows more than 70% enhancement in tryptophan over the original hybrid. In the state trials of Uttarakhand under organic condition, this hybrid gave more than 2.4% higher grain yield and was released for the state of Uttarakhand, India, in the year 2012 for commercial cultivation by the State Varietal Release Committee, Uttarakhand, for the hill conditions (Fig. 7.3) (Agrawal et al. 2015).

Table 7.2 MAS-derived single-cross QPM hybrids released for commercial cultivation in India

S. No.	Cultivar	Original Hybrid	Endosperm and Grain Color	Institute	Year
1.	Vivek QPM 9	Vivek Hybrid 9	Hard and Yellow	VPKAS, Almora	2008
2.	Vivek QPM 21	Vivek Hybrid 21	Hard and Yellow	VPKAS, Almora	2010
3.	Pusa HM4 Improved	HM4	Hard and Yellow	IARI, New Delhi	2017
4.	Pusa HM8 Improved	HM8	Hard and Yellow	IARI, New Delhi	2017
5.	Pusa HM9 Improved	HM9	Hard and Yellow	IARI, New Delhi	2017

Fig. 7.2 A crop of Vivek QPM 9

Pusa HM4 Improved This QPM hybrid contains 0.91% tryptophan and 3.62% lysine, which are significantly higher than the popular normal non-QPM hybrids. It matures in 87 days with an average yield of 6.4 t ha^{-1}. This hybrid was developed at the ICAR-Indian Agricultural Research Institute, New Delhi, and released for commercial cultivation in the year 2017 for summer (*Kharif*) season in the states of Punjab, Haryana, Delhi, plains of Uttarakhand, and western region of Uttar Pradesh.

Pusa HM8 Improved This is a newly developed QPM hybrid rich in tryptophan (1.06%) and lysine (4.18%) which is more than double of the popular non-QPM hybrid. It yields 6.3 t/ha in 95 days. The hybrid was developed at the ICAR-Indian Agricultural Research Institute, New Delhi, tested for 2 years under All India

Fig. 7.3 Cobs of Vivek QPM 21

Coordinated Research Project on maize and released for commercial cultivation in summer (*Kharif*) season in the states of Maharashtra, Andhra Pradesh, Telangana, and Tamil Nadu of Indian Union in the year 2017.

India is one of the first few countries to focus on *o*2 maize and released three *o*2 composites, namely, Shakti, Rattan, and Protina in 1970 (Dhillon and Prasanna 2001) and one modified superior *o*2 composite "Shakti 1" in 1997. Later on, India released several QPM hybrids *viz*., Shaktiman-1 (a three-way cross hybrid), Shaktiman-2, Shaktiman-3, Shaktiman-4, HQPM-1, HQPM-5, and HQPM-7 (single cross hybrids). Vivek QPM 9 was the first MAS-derived QPM hybrid released in the year 2008. It was followed by the release of another four QPM hybrids (Agrawal and Gupta 2010). Three popular hybrids were recently converted into QPM and were released for commercial cultivation in the year 2017. All the QPM composites and hybrids, with major traits released in India, are presented in Table 7.2.

7.5 QPM in Human and Animal Nutrition

The WHO (2007) suggested the daily requirement of 0.66 and 0.73 g protein/(kg BW•d) requirement for adults and infants, respectively. Correspondingly, the recommendations were also given for essential amino acids, namely, lysine (adult, 30 mg/(kg•d); infants, 35–45 mg/(kg•d)) and tryptophan (adult, 4 mg/(kg•d); infants, 4.8–6 mg/(kg•d)) (Institute of Medicine 2006). By virtue of two- to three-fold higher lysine and tryptophan content than non-QPM, QPM poses higher

biological value, balanced nitrogen index, and leucine/isoleucine ratio for better niacin synthesis (Ma and Nelson 1975; Vivek et al. 2008). The biological value depicts the amount of nitrogen retained in the body. The QPM poses exceptional biological value of 80% as compared to 40–57% in normal maize (Bressani 1992). The results were further confirmed by Abiose et al. (2015), where QPM-based diets showed better biological value (>60%) and digestibility (>60%) than the normal maize products. Owing to significant nutritional benefits, QPM could serve as an excellent source to overcome protein-energy malnutrition in humans and also better feed for livestocks. Additionally, replacement of common maize with QPM provides a more balanced protein source without scarifying energy, grain yield, and micronutrients and changing native food supply systems and economic benefits to farmers (Nuss and Tanumihardjo 2011).

7.5.1 QPM as Food for Human Being

Owing to the higher biological value of QPM, it was recommended as food to help in reducing protein deficiency. Accordingly, the QPM brought back the children suffering from kwashiorkor, a severe protein deficiency syndrome to normalcy in Columbia (Bressani 1990). QPM can also be used as an ingredient in the preparation of composite flours to supplement wheat flour for chapatti, bread, and biscuits preparation. In many countries such as in many countries such as Brazil, Zambia, Zimbabwe, and Ghana ten percent maize flour has been used in composite flours. According to a recent study in rural Ethiopia on the nutritional benefits of QPM and its acceptance as a food, when QPM was supplemented in food as their main starchy staple, the heights and weights of preschool children increased more than 20% faster than those of children who ate conventional maize. Maize is becoming a major staple food in Ethiopia as the price of tef and mdash, the traditional indigenous food, is rising beyond the means of resource-poor consumers, and their yields generally fall short of household needs. Rural communities rely more and more on maize for both calories and protein, especially where people lack access to other protein sources (Anon 2008).

Six-month feeding study of QPM and non-QPM conducted on children at Rajendra Agricultural University, Pusa of Bihar state in India, revealed increased body weight and arm circumference of children fed with QPM which was remarkably higher as compared to children fed with non-QPM (Mamatha et al. 2017). Similarly, in Uttar Pradesh another state of India, a QPM feeding experiment of 180 days on children (1–3 years age) revealed higher weight, height, head circumference, chest circumference, and arm circumference as compared to non-QPM, milk fed, and control diets (Chopra et al. 2011). On the other hand, studies conducted on children in Ethiopia revealed a positive effect on weight but no improvement for height (Akalu et al. 2010). A meta-analysis of community-based studies on consumption of QPM in Africa and India revealed that consumption of QPM instead of non-QPM leads to increase in the growth rate of 12% and 9% for weight and height, respectively, in infants and young children with mild to moderate undernutrition

(Gunaratna et al. 2010). Sixteen weeks of supplementation of QPM-based composite diets resulted in significant improvement in height, weight, weight-for-age, height-for-age, and weight-for-height z-scores and hemoglobin levels among the children receiving such diet with QPM (Maseta 2016). The consumption of QPM not only make the target population nutritionally sufficient but economically viable too.

A general conclusion about QPM is that it is superior to normal maize on its amino acid balance and nutrient composition and could improve the performance of various animals. It is more economical to use diets incorporating QPM since there will be a reduction in the use of fish meal and synthetic lysine additives. While studying the nutritional factor of QPM, together with soybean flour, brown sugar, banana meal, and oat meal, for Fisher rats, observed that QPM showed a good potential for utilization in nutritional supplements, especially when associated with soybean flour.

7.5.2 QPM as Feed in Poultry and Livestock

Maize is well accepted as the king of the feed ingredients. It is a primary source of energy supplement in animal diets and can contribute up to 30, 60, and 90% of the diet's protein, energy, and starch, respectively (Dado 1999). About 70–80% of maize production is used as a feed ingredient in the world. While comparing the feed value of normal maize (NM) and quality protein maize (QPM), Zhai (2002) reported that there was no significant difference on gross energy (GE), apparent metabolizable energy (poultry), and apparent digestible energy (pig) content between QPM and NM. Although NM had a higher GE content than QPM, its contents of AME (apparent metabolizable energy) for poultry and ADE (apparent digestible energy) for pig were lower than those of QPM. This indicates that the energy availability of QPM is little higher than that of NM.

Many investigations show superiority of QPM as source of feed for dairy and piggery. Although, chemical composition of fodder from QPM was similar to that of the normal maize, fodder of QPM varieties (HQPM 5, HQPM 7, and HQPM 9) showed better in vitro digestibility values (IVDMD% and IVOMD%) than other normal maize varieties (Vaswani et al. 2015). Studies conducted at International Livestock Research Institute, Debre Zeit Research Station, in Ethiopia, showed that QPM silage-based diet was superior in dry and organic matter degradability and had higher ammonia and volatile fatty acids concentration than normal maize stover-based diet (Tamir et al. 2012).

7.5.2.1 QPM as Feed for Layers

Zhai (2002) found that replacement of NM by QPM significantly enhanced the egg production. The QPM-based diets also increased the feed intake of the birds remarkably. The utilization of QPM in laying hen also enhanced the yolk color pigmentation. However, no significant effect was found on the weight of the eggs. Similarly, Osei

et al. (1999) reported that when QPM was added to pullet diets, the protein level can be reduced to 14% without any adverse effects on their performance, whereas, the addition of QPM to layer diets has significant effects on the age at first egg production, 50% hen day and housed production. This indicates that the QPM when used in feed result in considerable financial benefits without sacrificing performance.

7.5.2.2 Nutritional Superiority of QPM in Broilers

Osei et al. (1998) conducted two experiments, each lasting 6 weeks, to evaluate QPM as a feed gradient for broiler chickens. In Experiment 1, either normal hybrid maize or QPM was used as the sole source of protein and amino acids, and the diets were balanced for vitamins and minerals by the addition of a vitamin-mineral premix. These diets were compared with a balanced 21% crude protein starter-finisher broiler diet. In Experiment 2, broiler chicks were fed with combined starter-finisher diets of varying protein contents in which QPM replaced normal maize. The aim was to assess the potential for reducing dietary fish meal incorporation as QPM replaced normal maize. In both the experiments, feed and water were supplied ad libitum. The parameters measured included feed intake, growth rate, feed conversion efficiency, and carcass characteristics. The economics of broiler production was determined in Experiment 2. In Experiment 1, birds receiving QPM as the sole source of amino acids performed significantly better than their counterparts fed on normal maize. QPM-fed birds weighed an average 708.0 g each at the end of the trials compared with 532.0 g for those on normal maize. The corresponding feed efficiencies were 4.28 and 6.55, respectively. Compared with birds on the balanced diet, however, QPM was inadequate in supporting broiler growth. In Experiment 2, the use of QPM allowed the fish meal to be reduced from 19.5% to 13.5% without adversely affecting performance. They also found it cheaper to produce broilers using QPM than using normal maize. Similarly, Bai (2002) reported that the dietary replacement of normal maize by QPM significantly increased weight gain of days 21–42, 42–49, and 1–49 in broilers. They concluded that using QPM to replace normal maize in the broilers diet may have economic benefit due to improved weight gain, FCR, and decreasing of dietary lysine supplementation. In another study, dietary replacement of non-QPM with 50% QPM resulted significant improvement in body weight gain, feed conversion ratio, immune response, relative bursa weight, and breast muscle yield and lowered abdominal fat content of broilers (Panda et al. 2010). The feeding of QPM over non-QPM commercial hybrid *Nithyasri* in broiler chickens resulted improved breast meat yield and serum biochemical profiles and reduced the abdominal fat content and serum cholesterol (Panda et al. 2011).

7.5.2.3 Nutritional and Biological Superiority of QPM in Pigs

Nutritional studies of QPM has also been made in pigs. In a study by Zhai (2002), it was observed that the QPM had higher apparent and true ideal amino acid digestibility of most amino acids than those of NM in pigs. QPM had not only a higher

content of lysine but also higher digestibility of lysine for pigs. The reason for this increased digestibility is possibly due to increase of albumins/globulins ratio. However still methionine is one of the limiting amino acids in maize (both QPM and NM) when used as animal feed. Gao (2002) made a similar study in pigs and reported that in grower phase (20–50 kg), replacement of NM by equal ratio of QPM in pig diets significantly improved the average daily gain (ADG). This indicates that QPM as a feed is better than NM. This is due to higher lysine content and better digestibility of critical essential amino acids. However, replacement of NM by QPM had no significant effect on carcass characteristic of pigs. A QPM feeding trial on weaner pigs showed higher feed conversion efficiency of weaner pigs fed with QPM diet and also cheaper cost of production owing to QPM-based protein source (Mpofu et al. 2012). In addition to the direct impact on metabolism of animals, QPM can serve as an excellent substitution for high-value ingredients in feed (soybean meal) diets without adverse effect on animal performance, which helps in reducing cost of animal rearing (Prandini et al. 2011). Similarly, studies on the impact of QPM diet on growth and performance of weaner rabbits revealed that substitution of QPM could reduce the cost of weaner rabbits raising without affecting the growth and metabolism (Omage et al. 2009).

7.5.2.4 Economic Impact of QPM as Feed

Miguel and Pereira from CIMMYT, Mexico, evaluated pig and poultry feed cost and composition effects from including QPM as an alternative energy and protein source. Cost savings could be approximately 3.4% (about $5/ton) for pig feed, with QPM constituting about 80% of the ration and replacing all regular maize and synthetic lysine and 40% of soybean meal. Savings are slightly lower for poultry feed. However, if a 5% price premium for QPM over regular maize is assumed, most of the savings are lost, indicating that QPM should compete at the same price to be economically attractive as a commercial feedstuff (www.cimmyt.org).

7.6 Challenges in Large–Scale Adoption and Area Expansion of QPM

7.6.1 Constraints in Area Expansion Under QPM

QPM is a cost-effective way to improve the diets of protein-malnourished populations, where maize is one of the staple intakes. Several QPM cultivars have been developed in various national and international maize breeding programs and they are being disseminated and promoted. Some of the countries witnessed impressive adaption of QPM from farming community and better consumer acceptance. For instance, in Africa, Ghana is leading the QPM adoption and production with 70,000 ha of QPM area with the vast majority of *Obatanpa* variety (Krivanek et al.

2007). By 2009, more than ~40 countries had released QPM varieties for general cultivation (Sofi et al. 2009). China leads the Asian maize-growing countries, and interest in QPM has grown tremendously with active support of government. More than 100,000 ha are currently planted with QPM hybrids. It is expected that more than 30% of the total maize-growing area in China will be covered by QPM hybrids by 2020 (De-quan and Shihuang 1994). Mexico accounting around 2.5 million hectares of QPM and countries in Africa such as Mali, Burkina Faso, Benin, Ethiopia, Uganda, Mozambique, and South Africa are growing QPM in 5–20 thousand hectares. In developing countries, the QPM varieties have been contributing more than $1 billion annually (Vasal 2002; Nedi et al. 2016). However, despite of numerous QPM varieties released and diverse reports showing the importance of producing QPM on nutritional well-being of humans and farm animals, the adoption has been limited worldwide. Presently, QPM varieties are grown on roughly nine million acres worldwide, which encompass only <1% of the total area under maize production (Rugema 2014). The challenges facing dissemination of the QPM hybrids in the farmer's field includes seed availability, segregated procurement and marketing, procurement-to-consumption chain, policy support for premium prices (to QPM), and policy support to take QPM to consumption chain.

7.6.2 Awareness on Health Benefits of QPM Among Farmers and Consumers

In spite of the fact that the QPM technology is available to the farming community and consumers, majority of the consumers and farmers in developing and underdeveloped nations are not aware of the nutritional benefits of QPM. Intensive awareness campaigns needs to be initiated for making QPM popular among the farmers as well as consumers. Studies conducted in Africa revealed familiarity of several group of farmers to QPM even if they had minimal awareness about its nutritional benefits (De Groote et al. 2016). Approximately 94% of the respondents agreed to consume nutritionally superior yellow maize over white on knowing the health benefits of QPM (Stevens and Winter-Nelson 2008). In addition to spreading of awareness, health benefits of QPM should be spread for the QPM products to increase the consumer preference over conventional maize products.

7.6.3 Incentives and Policy Support to the QPM Stakeholders

Adequate policy supports and incentives from the respective governments are necessary for the success of programs on QPM. Presently, there are no incentives available for the farmers growing QPM and food processing industries using QPM. Therefore, the promotion of QPM requires incentives for QPM farmers and

food processing industries in some or other form. Subsidy for the cost of the QPM seeds and premium price for the QPM grains are viable propositions. The delivery of QPM to target population could be facilitated through food and feed processing industries. Midday meal and "public distribution system" are the other means to reach the QPM products to consumers. Processing and value addition to QPM products need to be encouraged.

7.6.4 Successful Public–Private Partnership

Effective public-private partnership can help in bringing magical adoption of QPM. It should include partnership in development of cultivars, commercialization, processing, value addition, and value chain development. The help of local non-governments, non-governmental organizations, and village self-help groups will be highly useful in this endeavour. In view of the successful spread and adoption of maize hybrids in India during the last two decades during which private sector played major role. It is imperative to rope in private sector for development of maize hybrids whose productivity is at par with that of the normal single cross hybrids. Hand-in-hand, seed production and dissemination of these QPM hybrids should also involve the private sector. In addition, utilization of QPM in preparation of various edible products like breakfast cereals, nachos etc. by the industry, can be accelerated by bringing them on board with the producers and researchers who can, in turn meet the requirement of various parameters of QPM grains.

7.6.5 Adequate and Organized Food Processing Industry

The well-informed consumers were found willing to purchase QPM-derived quality food products with premium price. In Kenya, maize consumers were willing to pay a premium price for biofortified maize (De Groote et al. 2011). Similarly, consumers were observed to pay 20–70% premium price for the biofortified foods developed through genetic engineering (De Steur et al. 2015). QPM-derived foods are expected to have a good demand if consumers are made aware of product from QPM. However, in most of the developing nations, processing facility and related marketing of QPM products are not well organized.

7.6.6 Strong Extension Programs and Creation of "Nutri-Village" Clusters

Strong extension program for the expansion of area under QPM and its acceptability among the consumers is required. Adaptation of strong extension programs could lead to expansion of QPM area up to 70% in Uganda and 30% in Tanzania (De

Groote et al. 2016). Involvement of local peoples, volunteers, and folk artist along with regular extension workers to create awareness about QPM among villagers using mass communications, community drama, road shows, and "field days" could increase its acceptability. Another viable approach for the popularization of QPM is the creation of "Nutri-village" clusters to ensure quality QPM seed production and QPM grains for the industries. The "Nutri-village" should be designed for production of QPM only. They may also be encouraged to go in the mode of "contract farming" for reasonable income of QPM farmers and continuous supply of QPM products for industries. It may be in the line of "Orange day" of Mozambique and Uganda for promotion of orange sweet potato.

An effort was jointly made by VPKAS, Almora, and Hindustan Insecticide Limited (company) by producing around 2000 quintals of QPM seeds involving 168 farm families in the Krishna Nagar, Nadia district of West Bengal, India. This is one of the successful examples for the large-scale production of public sector QPM hybrid, Vivek QPM 9, in seed village hub. The produce was used as seeds in the Northeastern states and West Bengal, in India.

7.6.7 Linking All the Stakeholders on Common "QPM Platform"

QPM has turned out to be an important discovery that has benefitted many African countries in reducing protein malnutrition significantly; however, technology has not been utilized fully in Asis in general and south Asia in particular where protein malnutrition is a major challenge. In order to harness the potential of QPM in Asis, there is a need to bring all the stakeholders like researchers, farmers, industries, seed companies, public organizations, NGOs, and SHGs for successful production, processing, value addition, and marketing of QPM. Support among the stakeholders will help in complementing and supplementing each other for the success of QPM in Asia.

7.7 Way Forward and Conclusions

QPM is one of the practical solutions to fight protein-energy malnutrition and has successfully been deployed in several African countries where maize is a staple food. It has been able to create an impact in reducing protein malnutrition in large population when the acceptability and support to farmers and consumers were available. QPM can, thus, be presented and supported as a better alternative to combat the malnutrition in the regions where maize constitutes a large part of the diet. Many developing countries in Africa and South America have maize as the staple food. Besides, QPM could also be popularized as a source of food among the Asian countries where maize is majorly preferred as animal and poultry feed. In these areas, there are pockets in which maize is consumed as food. Thus, replacing normal

maize with QPM is likely to have salutary impact as seen in Africa. Once a demand for QPM is created, it will help in diversifying cultivated lands from rice to maize especially in upland areas where rice productivity continues to hover around 1.00 t ha^{-1} even today. Furthermore, QPM enriched with Fe, Zn, and provitamin A can be used to overcome hidden hunger along with protein malnutrition (Gupta et al. 2015a, b; Mallikarjuna et al. 2014, 2015; Mallikarjuna 2015). Likewise, in combination with low phytate content, QPM will help in reducing Fe and Zn deficiency. Such a maize cultivar can be called as "super QPM" satisfying protein and micronutrient malnutrition in holistic manner. Recent advances like genome editing (CRISPR/Cas), genomic selection, and other molecular breeding will help in developing "super QPM."

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Chapter 8
Genetic Improvement of Basmati Rice: Transcendence Through Molecular Breeding

A. K. Singh, S. Gopala Krishnan, R. K. Ellur, M. Nagarajan, K. K. Vinod, P. K. Bhowmick, and B. Haritha

Abstract Basmati rice from the foothills of Himalayas is a connoisseurs' delight. Basmati rice improvement began with germplasm collection and pureline selection among the landraces. Systematic Basmati rice improvement program at ICAR-Indian Agricultural Research Institute, New Delhi (ICAR-IARI), led to a breakthrough with the release of first semidwarf high-yielding variety Pusa Basmati 1. Subsequently, the release of Pusa Basmati 1121, unique for its cooked kernel length and volume expansion, brought a significant change in Basmati cultivation. The total volume of Basmati rice exports quadrupled leading to 6.8 times increase in foreign exchange earnings during 2013–2014 (Rs. 29,299 crores). Furthermore, the development of Pusa Basmati 1509, the first early maturing Basmati rice variety, demonstrated that undesirable linkage between duration and yield can be broken, thus achieving better per day productivity while increasing profitability. Although better productivity and quality were achieved, these varieties were susceptible to various biotic and abiotic stresses. The beginning of the twenty-first century witnessed rice genome sequencing, which enabled the use of molecular tools for precision breeding in Basmati rice. Molecular marker-assisted selection has been effectively used in combination with phenotypic selection to develop Basmati rice varieties with resistance to diseases like bacterial blight and blast. The bacterial blight resistance genes *xa13* + *Xa21* were incorporated into the genetic background of Pusa Basmati 1, Pusa Basmati 1121, and Pusa Basmati 6 to develop Improved Pusa Basmati 1, Pusa Basmati 1718, and Pusa Basmati 1728, respectively. Similarly, Pusa Basmati 1637 carrying blast resistance gene *Pi9* in the genetic background of Pusa Basmati 1 was also developed. In addition to the release of varieties, molecular breeding has helped in creating several genetic stocks in Basmati rice with resistance to biotic stresses

A. K. Singh (✉) · S. Gopala Krishnan · R. K. Ellur · P. K. Bhowmick · B. Haritha
Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India

M. Nagarajan · K. K. Vinod
Rice Breeding and Genetic Research Centre, Division of Genetics, ICAR-Indian Agricultural Research Institute, Aduthurai, India

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such as BB, blast, sheath blight, and bakanae and tolerance to abiotic stresses such as salinity and drought. The high-yielding Basmati rice varieties developed have brought in a paradigm shift in Basmati rice cultivation, domestic consumption, and export to the tune of Rs. 25,000 crores annually, bringing prosperity to millions of Basmati rice farmers in the Indo-Gangetic Plains (IGP); this success story can be appropriately described as "Basmati revolution." This chapter describes five decades of concerted research efforts on genetic improvement of Basmati rice at the ICAR-IARI, India.

Keywords Basmati rice · Breeding · Geographical indication · Germplasm · Grain and cooking quality · Marker-assisted selection · Stresses · Variety

8.1 Introduction

Basmati is a specialty rice native to foothills of Himalayas in the Indo-Gangetic Plains (IGP). It is highly priced in the international market for its unique and well-balanced combination of kernel shape, pleasing aroma, fluffy cooked rice with high volume expansion, better linear kernel elongation with minimum swelling, good palatability, easy digestibility, and longer shelf life (Singh et al. 1988). Basmati rice has been conferred with GI tag (Geographical Indication No. 145 through certificate number 238 on 15 February 2016) for cultivation in the six states of India, namely, Punjab, Haryana, Himachal Pradesh, Uttarakhand, and Jammu and Kathua districts of Jammu and Kashmir and 27 districts of western Uttar Pradesh (Singh et al. 2018c). Genetic improvement in Basmati rice begun with pureline selection within landraces, which led to development of several varieties (Table 8.1); some of the landmark traditional Basmati varieties are described as under.

Table 8.1 Traditional Basmati rice varieties released for commercial cultivation

Variety	Notification no. and date	Parentage	Developed at
Basmati 217	SO.4045, 24.06.1969	Pureline selection from local Basmati germplasm	Rice Research Station, Kapurthala (PAU)
	SO.361, 30.06.1973		
Basmati 370	SO.361, 30.06.1973	Pureline selection from Dehradun Basmati	Govt. Agri. Farm, KalashahKaku (Now in Pakistan)
	SO.786, 02.02.1976		
Type 3	SO.13, 19.12.1978	Pureline selection from Dehradun Basmati	Rice Research Station, Nagina
Taraori Basmati	SO.1(E), 01.01.1996	Pureline selection from Karnal Local	Rice Research Station, Kaul
Ranbir Basmati	SO.1(E), 01.01.1996	An early mutant of Basmati 370	Regional Agricultural Station, RS Pura, Jammu
Basmati 386	SO.647(E),09.09.1997	Pureline selection from local Basmati germplasm	Rice Research Station, Kapurthala (PAU)

8.1.1 Basmati 370

In 1921, a total of 1265 rice germplasm were evaluated at Rice Research Station at Doiwala in Dehradun along with the local Basmati collections. The Basmati rice from Dehradun Valley became popular in the trade circles as Dehradun Basmati (Singh and Singh 2009). However, the first systematic Basmati rice research improvement has its origin at Rice Research Station, Kala Shah Kaku (part of erstwhile united India and now in Punjab province of Pakistan). A large number of Basmati rice genotypes collected from across the Terai region were evaluated, and seven promising strains of Basmati were identified. One among these, strain "370/27" was released as "Basmati 370" for commercial cultivation in 1933, which was popular in the export markets until the late 1980s (Ramaiah and Rao 1953).

8.1.2 Taraori Basmati

During 1981–1984, a collection of 200 Basmati rice germplasm was evaluated at Regional Rice Research Station, Kaul, of CCS Haryana Agricultural University, Hisar. One of the germplasm lines namely, Haryana Basmati Collection 19 (HBC19) was identified promising with superior cooking quality and strong aroma, which was later released for commercial cultivation in 1996 as Taraori Basmati. This variety is still used as a national check for Basmati quality traits in the National Basmati Trials.

However, traditional Basmati rice genotypes were tall, susceptible to lodging, photo- and thermosensitive, as well as poor yielders (Singh and Singh 2009). There was less genetic gain achieved through pureline selection. It was felt necessary to improve the productivity of Basmati rice varieties through hybridization. This necessitated understanding the genetics of the Basmati quality traits.

8.2 Genetics of Basmati Quality Traits

During the early 1970s, pioneering basic research carried out at ICAR-Indian Agricultural Research Institute, New Delhi (ICAR-IARI), led to standardization of protocols for estimating various Basmati grain quality parameters and analyzing their inheritance pattern (Singh et al. 2004). A simple sensory evaluation method for quick and efficient assessment of aroma was developed (Sood and Siddiq 1978), which involved adding 10 ml of 1.7% KOH solution to a small Petri plate containing about 2 g of finely minced sample of green leaf or stem and recording observation on the aroma. This is one of the most effective methods used till date to screen large number of breeding material for aroma within a short span of time. Similarly, a method for estimation of kernel elongation and mechanism of kernel elongation

was also worked out. In one of the first reports, scanning electron microscopy was used for studying the mechanism of kernel elongation, which found that as against nearly equidistant penta- or hexagonal cells arranged in a bee-comb fashion in the elongating types, the cells were found to be long, rectangular, and arranged radially in columns extending from the center to the periphery in the breadthwise swelling types. These protocols along with set of other methods standardized for estimation of amylose, alkali spreading value (ASV), etc., at International Rice Research Institute, Philippines, helped in understanding the genetic basis of Basmati grain quality characters.

Further, it was concluded that aroma in Basmati rice is governed by a single recessive gene (Sood and Siddiq 1978) and all other traits such as milled rice length, kernel elongation after cooking, and volume expansion are polygenic in nature.

8.3 Era of High-Yielding Rice Varieties

Simultaneously, in the mid-1960s, the introduction of semidwarfing gene, *sd1*, into rice genotypes spurred in the green revolution in India. With the improvement in productivity achieved through the introduction of IR8 and further development of semidwarf varieties with better yields, the rice production from Punjab and Haryana improved manifolds. However, in order to make Basmati rice farming more remunerative, Prof. M.S. Swaminathan envisaged that there is a need to combine quality attributes of traditional Basmati rice varieties in the high-yielding background (Siddiq et al. 2012). Based on this, a systematic breeding program on genetic improvement of Basmati rice was initiated at the ICAR-IARI in 1966, which has led to the development of several high-yielding Basmati rice varieties (Singh and Singh 2010) as described below.

8.3.1 Pusa Basmati 1: The Primer for Basmati Revolution

With the understanding of genetics of Basmati quality traits and the introduction of semidwarfing gene, a convergent breeding approach involving a high-yielding non-aromatic semidwarf variety, Taichung Native 1, and several other rice varieties along with the Basmati rice variety, Basmati 370 (Singh et al. 2018c), were adopted to develop Pusa 150. Further, a cross of Pusa 150/Karnal Local led to development of a world's first semidwarf, photoperiod insensitive and high-yielding Basmati rice variety Pusa Basmati 1 (PB 1) which was released for cultivation during 1989. This variety is characterized by extra-long slender aromatic grains, higher linear cooked kernel elongation with potential grain yield of 6–7 t/ha, and medium early duration (135–140 days seed to seed maturity) which made PB 1 the most sought variety by the farmers, exporters, and consumers. The release of PB 1, thus, revolutionized the Basmati rice production in India during the mid-1990s.

During 1995–2007, this variety contributed nearly 60% of the total Basmati rice export value which is ~Rs. 14,000 crores and brought prosperity to Basmati rice farmers of Punjab, Haryana, western UP, and Uttarakhand. PB 1 has been extensively used as a donor for Basmati quality traits and high yield in the national Basmati breeding program (Singh et al. 2018c).

8.3.2 Pusa Basmati 1121: Setting New Standards for Basmati Quality

Selective inter-mating of advanced breeding lines, namely, Pusa 614-1-2 and Pusa 614-2-4-3, led to the development of Pusa 1121, a genotype with exceptionally high cooked kernel and volume expansion, which was released for Delhi state in 2003 (Singh et al 2002). Until 2008, as per requirement of the Commerce Ministry, for a variety to qualify as Basmati, it was necessary that it possessed, in its pedigree, one of the traditional Basmati rice varieties as an immediate parent. However, during 2008, the Basmati quality was redefined based on Basmati quality parameters, linking it with geographical indication (GI) to make Basmati breeding more inclusive in terms of developing new varieties with resistance to diseases and insect pest. Following the new definition of Basmati standards, Pusa 1121 was notified as Pusa Basmati 1121 (PB 1121) in 2008 and released for the states of Punjab, Haryana, and Delhi. PB 1121 is a semi-tall rice variety with a seed to seed maturity of 140–145 days and yield potential of up to 5.5 t/ha. It combines unique Basmati quality with an extra-long slender (9.00 mm) aromatic grains with 54.5% head rice recovery and very occasional chalky grains (Singh et al 2018a). This variety possesses highest kernel length after cooking (21.0–21.5 mm), exceptionally high cooked kernel elongation ratio of 2.70, volume expansion more than four times, and intermediate amylose content.

Box 8.1 Minimum Standards for Basmati Rice Variety

With better understanding of the genetics of Basmati quality, the genes governing key Basmati quality traits, and the refinement in the screening protocols for Basmati quality parameters, the Ministry of Agriculture, Government of India, has established minimum standards for the rice varieties to qualify as a Basmati variety, notified vide OM F. No. 17–12/2007-SD. IV, Government of India dated 29 May 2008:

1. The variety, either traditional known Basmati or evolved through breeding process, should be tested through National Basmati Trials (NBT) of All India Coordinated Rice Improvement Project, Hyderabad, and released/notified under the Seed Act 1966 of India (54 of 1966), and amendment made therein.
2. The variety should be suitable for cultivation in the GI region earmarked for Basmati rice cultivation.

(continued)

3. It should fulfill the quality parameters of primary and ancillary characters as a prerequisite that has to be verified by one or more laboratories identified by the Indian Institute of Rice Research (formerly Directorate of Rice Research), Hyderabad, ICAR, for the purpose.
4. The variety should be proposed for release/notification with the term "Basmati" in the body of the denomination, along with its initial evaluation trial (IET) number in parenthesis.

Standards for Basmati rice qualification under National Basmati Trials

S. No.	Parameters[a]	Value
Main characters		
1	Minimum average precooked milled rice length (mm)	6.61
2	Average precooked milled rice breadth (mm)	≤2.00
3	Minimum length/breadth ratio of precooked milled rice (L/B ratio)	3.50
4.	Minimum average cooked rice length (mm)	12.00
5.	Minimum cooked elongation/precooked rice length ratio	1.70
6.	Average volume expansion ratio	>3.5
7.	Aroma	Present (quality sensory analysis as panel test*)
8.	Texture of cooked grain: high integrity (without bursting), non-stickiness, tenderness, good taste, and good mouth feel	Present (quality sensory analysis as panel test*)
Ancillary characters		
1	Amylose content range	20–25%
2	Alkali spreading value (ASV) range	4–7
3	Minimum brown rice recovery (%)	76%
4	Minimum milled rice recovery (%)	65%
5	Minimum head rice recovery (%)	45%

[a]The grain sample for analyses will necessarily have to be "aged" for 3 months under protected conditions at normal temperature as milled kernel;
*Qualitative sensory analysis as Panel Test as per standardized protocol (ICAR-Indian Institute of Rice Research, Hyderabad)

Owing to its unique grain and cooking quality, PB 1121 is the most preferred Basmati rice variety in the global market due to which Basmati rice area increased from 0.78 mha in 2004 to 2.12 mha in 2015, in which PB 1121 was grown in 70% of the total area under Basmati cultivation (Singh et al. 2018c). This variety alone contributed 4.39 mt of milled rice, accounting for ~68% of the total Basmati rice production. The average net income through the cultivation of PB 1121 was estimated to be ~US$ 1400/ha as compared to US$ 650/ha from other Basmati rice varieties such as Basmati 370 and Taraori Basmati in 2017 (Singh et al. 2018c). Since its release, this variety alone has earned around US$ 20.80 billion (~ Rs. 1.5 lakh crores) through exports and domestic consumption spurring a Basmati rice revolution in India (Singh et al. 2018c).

8.3.3 *Pusa Basmati 6: Combining Higher Yield with Typical Basmati Quality*

Pusa Basmati 6 (PB 6) is another semidwarf high-yielding (6–7 tons/ha) Basmati rice variety with sturdy stem released for commercial cultivation in 2008. It was developed from the cross, PB 1/PB 1121. It is known for its unique uniform cooked kernel shape, strong aroma, and translucent grains with less than 4% chalky grains. This variety is very popular in Sirsa and Fatehabad districts of Haryana and Gurdaspur, Amritsar, Muktsar, Patiala, Ludhiana, and Sangrur and Fatehgarh Sahib districts in Punjab.

8.3.4 *Pusa Basmati 1509: The Climate Smart Basmati Rice Variety*

In 2013, ICAR-IARI released another landmark Basmati rice variety, Pusa Basmati 1509 (PB 1509), which combined early maturity, semidwarf plant stature, and Basmati grain quality for the first time (Singh et al. 2014). It was developed from the cross, PB 1121/Pusa 1301, and has been released for cultivation in Delhi, Uttar Pradesh, and Punjab. It has a potential yield of 7.0 t/ha in 120 days. It possesses extra-long slender grains (8.41 mm) with very occasional grain chalkiness, very good kernel length after cooking (19.1 mm) (Fig. 8.1), intermediate amylose content (21.2%), and strong aroma.

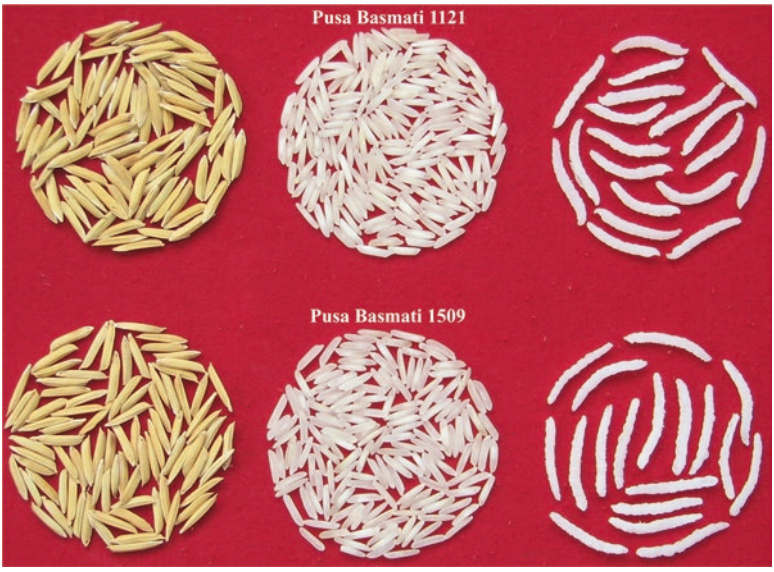

Fig. 8.1 Grain and cooking quality characters of PB 1121 and PB 1509

8.4 Impact of High-Yielding Basmati Rice Varieties

Altogether, the genetic improvement of Basmati rice at ICAR-IARI through conventional breeding involving hybridization and selection led to the development and release of four high-yielding Basmati rice varieties (Table 8.2), which has not only resulted in Basmati rice revolution but also created a unique profitable business model involving farmers, millers, traders, and consumers. This helped in enhancing the productivity from 2.5 t/ha to 6.0–8.0 t/ha while reducing the total crop duration from 160 days to 120–145 days (Fig. 8.2). The grain and cooking quality

Table 8.2 Basmati rice varieties developed through hybridization and selection

Variety	Pedigree	IET no.	Year of release	Duration (days)	Yield potential (t/ha)	Areas recommended
Pusa Basmati 1	Pusa 150/ Karnal Local	IET 10364	1989	135–140	5.0	Punjab, Haryana, Delhi, Western UP, Uttarakhand, Jammu and Kashmir
Pusa Basmati 1121	Pusa 614-1-2/ Pusa 614-2-4-3	IET 18004	2003	140–145	5.5	Punjab, Haryana, and Delhi
Pusa Basmati 6	PB 1/Pusa 1121-92-8-2-7-1	IET18005	2008	140–145	6.0	Punjab, Haryana, Delhi, Jammu and Kashmir, Uttarakhand, and western Uttar Pradesh
Pusa Basmati 1509	Pusa 1301/ PB 1121	IET 21960	2013	120	6.0	Delhi, Western UP, and Punjab

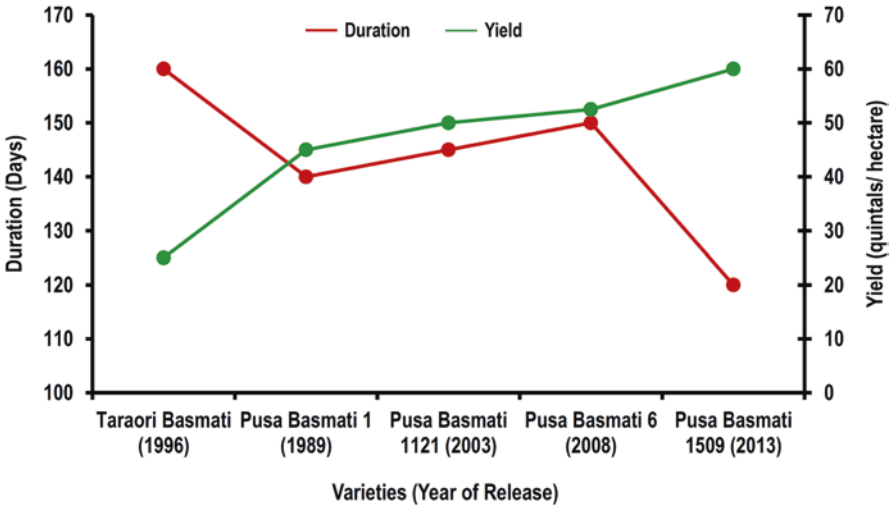

Fig. 8.2 Duration and yield of different Basmati rice varieties released since 1989

Table 8.3 Grain and cooking quality characteristics of improved Basmati rice varieties

Variety	Milling%	HRR%	KLBC (mm)	KBBC (mm)	KLAC (mm)	ER
Pusa Basmati 1	67.0	48.5	7.38	1.80	14.75	2.00
Pusa Basmati 1121	70.5	54.5	9.00	1.90	21.50	2.69
Pusa Basmati 6	66.7	57.3	7.31	1.73	16.07	2.19
Pusa Basmati 1509	68.1	49.5	8.19	1.86	18.20	2.22

HRR head rice recovery, *KLBC* kernel length before cooking, *KBBC* kernel breadth before cooking, *KLAC* kernel length after cooking, *ER* elongation ratio

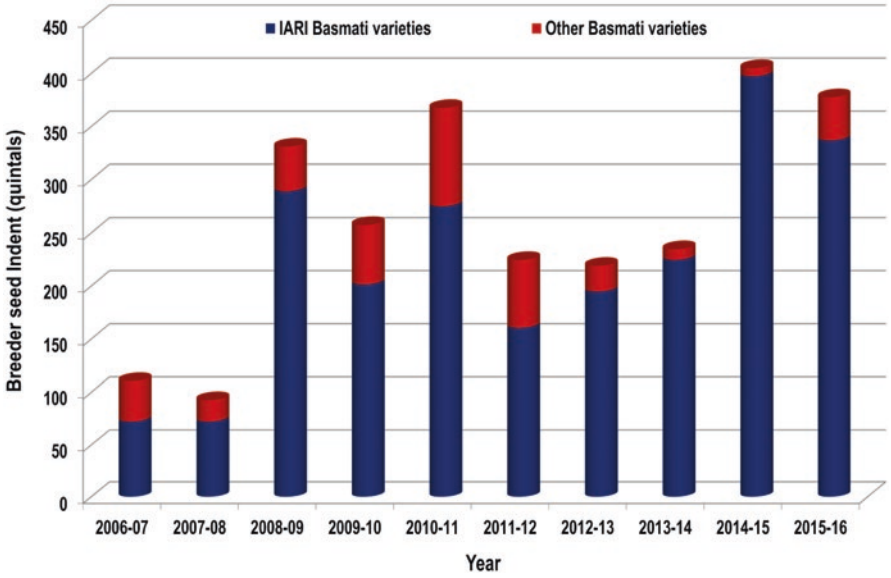

Fig. 8.3 Breeder seed indent of IARI Basmati rice varieties [PB 1, PB 1121, Improved PB 1 (since 2007), PB 6 (since 2008), and PB1509 (since 2013)] to the total Basmati rice breeder seed indent since 2006

characteristics of these Basmati varieties are presented in Table 8.3. The popularity of Basmati varieties developed by ICAR-IARI with the farmers is evident from the breeder seed indent of these Basmati rice varieties as compared to total Basmati rice breeder seed indent (Fig. 8.3).

8.5 Molecular Mapping of Basmati Quality Traits

To identify the genomic regions governing Basmati quality characters, several QTL mapping experiments were conducted. A major recessive gene *badh2* governing aroma in Basmati rice was mapped onto chromosome 8 which was further cloned

and functionally characterized (Bradbury et al. 2005). A 8 bp deletion in *badh2* was reported to be a functional region which leads to accumulation of the principle aromatic compound, 2-Acetyl-1-pyrroline (2-A,P), in Basmati rice. Another gene, *badh1*, located on chromosome 4 was also reported to govern aroma in Basmati rice (Singh et al. 2010). The grain length in Basmati rice is mainly governed by a major gene *GS3* on chromosome 3 with C to A mutation in exon 2 (Singh et al. 2011). Additionally, Amarawathi et al. (2008) mapped three QTLs governing grain length on chromosomes 1 and 7 in PB 1121. The markers associated with Basmati grain quality traits are available and are used routinely in marker-assisted improvement of Basmati rice (Anand et al. 2013; Anand et al. 2015).

Although conventional breeding through hybridization and selection has helped in developing high-yielding Basmati rice varieties, which are very popular among various stake holders, these varieties are highly susceptible to various biotic and abiotic stresses which affect their yield as well as quality.

8.6 Marker-Assisted Breeding of Basmati Rice Varieties with Resistance to Biotic Stresses

Among the biotic stresses, major diseases include bacterial blight (BB), blast, sheath blight, bakanae, brown planthopper (BPH), etc. (Singh and Gopala Krishnan 2016). Although the application of chemical pesticides is an option for management of these diseases and pests, but developing genetic resistance in Basmati rice varieties is an eco-friendly approach (Singh et al. 2012a, b). Till date, several genes/QTLs imparting resistance to BB (42 genes), blast (105 genes), sheath blight (several QTLs), bakanae (3 QTLs), and BPH (34 genes) have been identified. However, none of these genes were available in the Basmati rice germplasm. This limited their use in Basmati improvement program.

Large repertoire of genomic resources in rice (Gopala Krishnan et al. 2012) makes it possible to implement precision breeding through molecular marker-assisted selection, which provides a unique opportunity for transferring desirable genes from unadapted donors to otherwise agronomically superior Basmati rice cultivars to overcome the susceptibility to these biotic stresses (Singh et al. 2011). Marker-assisted backcross breeding (MABB) utilizing precise selection for target locus (foreground selection), screening for Basmati quality (phenotypic selection), and recovery of recurrent parent genome using genome-wide markers (background selection) (Singh et al. 2011) have been successfully employed for development of Basmati rice varieties with inbuilt resistance to major diseases such as BB, blast (Ellur et al 2016a; Ellur et al 2016b; Sagar et al. 2018), and sheath blight (Table 8.4), while efforts are underway to incorporate resistance to bakanae and BPH in Basmati rice (Singh et al. 2011). A brief description of Basmati rice varieties developed through MABB is given as under.

Table 8.4 Improved Basmati rice varieties with inbuilt resistance to biotic stresses developed through molecular marker-assisted breeding

Variety	Pedigree	Year of release	Duration (days)	Yield (t/ha)	Special attributes	Areas recommended
Improved Pusa Basmati 1 (IET 18990)	PB 1/ IRBB 55/ PB1*1	2007	135–140	5.0–5.5	Possesses bacterial blight resistance genes *xa13* and *Xa21* in the background of PB 1	Punjab, Haryana, Delhi, Western UP, Uttarakhand, Jammu and Kashmir
Pusa Basmati 1609 (IET 22778)	Pusa 1602/Pusa 1603	2014	120–125	6.0–6.5	Possesses two genes for blast resistance (*Pi2* and *Pi54*)	Punjab, Delhi, and Uttarakhand
Pusa Basmati 1637 (IET 24570)	PB 1/ IRBL 9-W/PB 1*3	2016	130–135	5.0–5.3	Possesses a broad-spectrum blast resistance gene, *Pi9* in PB 1 background	Punjab, Haryana, Delhi, Jammu and Kashmir, Uttarakhand, and western Uttar Pradesh
Pusa Basmati 1718 (IET 24573)	PB 1121/ SPS 97/ PB 1121*3	2017	140–145	5.0–5.3	Possesses two genes for bacterial blight resistance (*xa13* and *Xa21*) in PB 1121 background	Punjab, Haryana, and Delhi
Pusa Basmati 1728 (IET 24573)	PB 6/Pusa 1460/PB 6 *3	2017	140–145	4.5–5.0	Possesses two genes for bacterial blight resistance (*xa13* and *Xa21*) in PB six background	Punjab, Haryana, Delhi, Jammu and Kashmir, Uttarakhand, and western Uttar Pradesh
Pusa Basmati 1884[a]	Pusa 1726/Pusa 1727	2018	135–140	5.5–6.0	Possesses two genes for blast resistance (*Pi2* and *Pi54*) in PB six background	Punjab, Haryana, Delhi, Jammu and Kashmir, Uttarakhand, and western Uttar Pradesh

[a]Identified for release in 2018

8.6.1 Improved Pusa Basmati 1

Owing to the susceptibility to BB, the productivity of PB 1 was severely affected. Through marker-assisted breeding, two genes for BB resistance, namely, *xa13* and *Xa21*, were incorporated into PB 1 without compromising the Basmati quality (Joseph et al. 2004). A novel strategy of accelerating the recovery of recurrent parent phenotype through integration of phenotypic selection in the marker-assisted breeding was successfully demonstrated through the development of Improved PB

1 (Joseph et al. 2004). Background analysis was carried out using AFLP (Joseph et al. 2004) and SSR markers (Gopalakrishnan et al. 2008) to identify the families with maximum recurrent parent genome recovery. The improved Pusa Basmati 1 with resistance to BB was released for commercial cultivation in Punjab, Haryana, Delhi, Western UP, Uttarakhand, and Jammu and Kashmir in 2007 (Singh 2007a; Singh et al. 2007b).

Box 8.2 Formulation of Near-Isogenic Lines (NILs) Trials in AICRIPs
With the release of Improved Pusa Basmati 1, a NIL of PB 1, developed through marker-assisted breeding, there came another significant development in varietal testing in All India Coordinated Rice Improvement Project (AICRIP). Earlier, 3 years testing was necessary for release of any new variety. However, with the development of MAS-derived NILs, it was felt that 2 years testing should be sufficient to take a decision to release the NIL in target environment since the improved genotype is developed in the background of an already popular and widely cultivated variety. Accordingly, the near-isogenic lines (NILs) trial was formulated in AICRIP with a specific set of guidelines for evaluation. Based on the experience in rice, NIL trials were extended for testing MAS-derived NILs in other crops as well.

8.6.2 Pusa Basmati 1637: Blast-Resistant NIL of Pusa Basmati 1

Pusa Basmati 1637 is a NIL of PB 1 possessing *Pi9* gene for blast resistance developed through MABB. It has been released for commercial cultivation in the Basmati-growing regions of the western Uttar Pradesh, National Capital Region of Delhi, Uttarakhand, Haryana, and Punjab (Singh et al. 2017a). The field view of PB 1637 is presented in Fig. 8.4. PB 1637 has exhibited resistant reaction against blast disease with an SI of 2.8 as compared with its recurrent, PB 1, which showed highly susceptible reaction with SI of 6.3. It possesses long slender grains (7.3 mm) with very occasional grain chalkiness, very good kernel length after cooking (13.8 mm), and strong aroma. Annually, fungicides worth Rs. 50 to 60 crores are being used to control blast disease on Basmati crop. This variety being resistant to both leaf and neck blast would help in significantly reducing the use of tricyclazole for which the maximum residue level has been reduced recently to the minimum detectable level of 0.01 ppm. Thus the cultivation of PB 1637 will also economize cultivation cost and also minimizes the risk of pesticide residue, which is a major concern in both domestic and global Basmati trade.

Fig. 8.4 Field view of blast-resistant Basmati rice variety Pusa Basmati 1637

8.6.3 Pusa Basmati 1718: BB-Resistant NIL

Pusa Basmati 1718 is a MAS-derived near isogenic line of the popular Basmati rice variety, PB 1121, possessing two genes for BB resistance, namely, *xa13* and *Xa21*, developed through MABB. It has been released for the Basmati-growing states of Punjab, Haryana, and Delhi in 2017 (Singh et al. 2018b). It has a seed to seed maturity of 136–138 days and produces yield ranging from 5.0 to 5.3 t/ha but has produced potential yield of up to 6.04 t/ha. It is highly resistant to bacterial blight disease as compared to the recurrent parent, PB 1121, which is highly susceptible.

8.6.4 Pusa Basmati 1728: BB-Resistant NIL of Pusa Basmati 6

Pusa Basmati 1728 is a NIL of PB 6 with bacterial blight resistance governed by *xa13* and *Xa21* which were transferred through molecular marker-assisted breeding (Singh et al. 2017b). It has been released for Punjab, Haryana, Delhi, Jammu and Kashmir, Uttarakhand, and western Uttar Pradesh of the Basmati-growing region of India in 2016. PB 1728 has a seed to seed maturity of 140–145 days producing 4.5–5.0 t/ha and has a potential yield of up to 6.5 t/ha. It has shown highly resistant reaction (susceptibility index −2.8) as compared to the severe susceptibility shown by parent PB 6 (susceptibility index −7.0).

8.6.5 Pusa Basmati 1884: Blast-Resistant NIL of PB 6

Pusa 1884-3-9-175 is a MAS-derived NIL of a popular Basmati rice variety, PB 6, possessing two genes for blast resistance, *Pi2* and *Pi54*, with seed to seed maturity of 134–139 days with a yield of 5.5–6.0 t/ha. It has been identified for release in the Basmati-growing regions of the western Uttar Pradesh, Delhi, Uttarakhand, Haryana, and Punjab in the AICRIP workshop at Indian Institute of Rice Research, Hyderabad held in the year 2018.

Besides the development and release of Basmati rice varieties with BB and blast resistance, Basmati genotypes with multiple stress tolerance such as Pusa 1608 have been developed (Singh et al. 2012a, Singh et al. 2012b). It is the first successful example, where marker-assisted selection has been utilized for transferring of genes conferring resistance to three different diseases in rice wherein genes, *xa13* and *Xa21*, for BB resistance, *Pi54* for blast resistance, and a major QTL *qSBR11–1*. In a unique attempt, for the first time, a set of seven major blast resistance genes, namely, *Pi1*, *Pi54*, *Pita*, *Pi*-5, *Pib*, *Pi9*, *and Pi2*, from five different donors DHMAS70Q164-2a, IRBL5-M, IRBLb-B, IRBL9-W, and IRBLz5-CA have been transferred into the genetic background of PB1 through MABB (Khanna et al. 2015a, Khanna et al. 2015b). Two, three, and four blast resistance gene pyramids have been developed which will not only serve as an excellent resource for functional genomic analyses for blast resistance but also serve as valuable donors for blast resistance genes in Basmati rice improvement (Singh and Gopala Krishnan 2016). MABB was carried out to transfer seven blast genes governing resistance to blast disease of rice, respectively. Additionally, advanced backcross-derived lines with *Bph18*, *Bph20*, and *Bph21* in the genetic background of PB 1121 and Pusa Basmati 6 have been developed, which are in various stages of evaluation. More recently, marker-assisted introgression of QTLs governing bakanae resistance, namely *qBK1.2* and *qBK 1.1* (Fiyaz et al. 2016), and genes governing neck blast resistance is under progress in the genetic background of PB 1121 and PB 1509.

8.7 Marker-Assisted Breeding for Abiotic Stress Tolerance in Basmati Rice

8.7.1 Marker-Assisted Incorporation of QTLs for Salt Stress Tolerance into Basmati Rice

Rice is highly sensitive to salt stress at seedling and reproductive stages, and development of salinity tolerance at seedling stage is important for crop establishment (Vinod et al. 2013). PB1121, PB1, and PB 1509 are widely cultivated in Haryana occupying almost 0.5 mha (80%) of the Basmati rice area in the state, which is severely affected by inland salinity owing to brackish underground water used for irrigation. As these varieties are sensitive to seedling stage salinity stress,

incorporation of *Saltol*, a major QTL for seedling stage salinity tolerance (Gregorio et al. 1997), in these varieties was initiated. Near isogenic lines carrying *Saltol* in the background of PB 1121 (Babu et al. 2017), PB1 (Singh et al. 2018c), and PB 1509 (Yadav et al. 2017) have been developed, which are being evaluated for their performance and will help in improving Basmati rice yields under salt stress.

8.7.2 Marker-Assisted Breeding for Development of Herbicide-Tolerant Basmati Rice Suitable for Direct-Seeded Rice (DSR) Cultivation

With increasing water and labor scarcity, there is a shift from transplanted rice to direct-seeded rice (DSR). Though DSR is a boon to the farmers, it suffers from weed infestation, which necessitates the use of herbicides. Among different herbicides, the ALS inhibitor family, which includes sulfonylureas (SUs), imidazolinones (IMIs), triazolopyrimidines (TPs), pyrimidinyloxybenzoates (POBs), and sulfonylamino carbonyl triazolinones (SCTs), is considered safest as the ALS biological pathway exists only in plants and not in animals. On the contrary, the most commonly used herbicide in rice field, i.e., bispyribac sodium, has harmful effect on mammals if it gets in food/feed chain (Elalfy et al. 2017). However, rice crop is per se sensitive to ALS inhibitors. Thus, it requires to develop rice plant carrying a mutant allele of ALS, which is insensitive to ALS inhibitors. We have identified an EMS-induced mutant of *Als* gene, named HTM-N22, in the rice variety Nagina 22, with tolerance to the herbicide "imazethapyr" (Shobha et al. 2017). Further, MABB has been employed to transfer herbicide tolerance from HTM-N22 to PB 1121 and a set of advanced backcross PB 1121 progenies with resistance to imazethapyr are in advanced stages of testing (Grover et al. 2017).

8.7.3 Marker-Assisted Incorporation of QTLs Governing Tolerance to Reproductive Stage Drought Tolerance

Since all the Basmati rice varieties released till date have been bred for irrigated ecosystem with assured irrigations, they are highly susceptible to drought, which limits both yield and grain quality under drought stress. MABB was adopted to transfer a major QTL, *qDTY1.1*, governing grain yield under reproductive stage drought stress, from a non-Basmati rice drought-tolerant rice variety "Nagina 22" to Basmati rice variety Pusa Basmati 1 for enhancing grain yield under drought stress at reproductive stage, and superior backcross-derived progenies with significant yield advantage under reproductive stage drought stress have been identified, which will help in saving water as well as mitigating yield losses due to drought stress in Basmati rice (Dhawan et al. 2015; Gopala Krishnan et al. 2017).

8.8 Future Prospects

Breeding strategies for Basmati rice improvement has come a long way from simple selection from germplasm collection to pedigree breeding transforming in MABB to rectify the specific weaknesses of otherwise good varieties. Although the high-yielding dwarf varieties have brought a revolution in production and significant improvement in certain quality attributes such as volume expansion and elongation, there is still a need to further improve their aroma and taste. Therefore, understanding the molecular and biochemical basis of Basmati quality traits is a challenge for future. This would require whole-genome sequencing of the leading Basmati rice varieties such as Basmati 370, Taraori Basmati, PB1, PB1121, and PB1509 to understand the molecular basis of their principal features with respect to yield and quality traits. This would facilitate development of new generation Basmati rice varieties combining yield, quality, adaptation, resistance to biotic and abiotic stresses, and improved grain and nutritional quality as per farmers, consumers, and market needs.

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Chapter 9
Groundnut Entered Post-genome Sequencing Era: Opportunities and Challenges in Translating Genomic Information from Genome to Field

Manish K. Pandey and Rajeev K. Varshney

Abstract Cultivated groundnut or peanut (*Arachis hypogaea*) is an allopolyploid crop with a large complex genome and genetic barrier for exchanging genetic diversity from its wild relatives due to ploidy differences. Optimum genetic and genomic resources are key for accelerating the process for trait mapping and gene discovery and deploying diagnostic markers in genomics-assisted breeding. The better utilization of different aspects of peanut biology such as genetics, genomics, transcriptomics, proteomics, epigenomics, metabolomics, and interactomics can be of great help to groundnut genetic improvement program across the globe. The availability of high-quality reference genome is core to all the "omics" approaches, and hence optimum genomic resources are a must for fully exploiting the potential of modern science into conventional breeding. In this context, groundnut is passing through a very critical and transformational phase by making available the required genetic and genomic resources such as reference genomes of progenitors, resequencing of diverse lines, transcriptome resources, germplasm diversity panel, and multi-parent genetic populations for conducting high-resolution trait mapping, identification of associated markers, and development of diagnostic markers for selected traits. Lastly, the available resources have been deployed in translating genomic information from genome to field by developing improved groundnut lines with enhanced resistance to root-knot nematode, rust, and late leaf spot and high oleic acid. In addition, the International Peanut Genome Initiative (IPGI) have made available the high-quality reference genome for cultivated tetraploid groundnut which will facilitate better utilization of genetic resources in groundnut improvement. In parallel, the development of high-density genotyping platforms, such as Axiom_Arachis array with 58 K SNPs, and

M. K. Pandey (✉) · R. K. Varshney
International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India
e-mail: m.pandey@cgiar.org, r.k.varshney@cgiar.org

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constitution of training population will initiate the deployment of the modern breeding approach, genomic selection, for achieving higher genetic gains in less time with more precision.

Keywords Genomic resources · Trait mapping · Gene discovery · Diagnostic markers · Genomics-assisted breeding · Molecular breeding products

9.1 Introduction

Groundnut, also popularly known as peanut, is a globally important legume crop grown in >100 countries of the tropic and subtropic regions of the world. This crop is classified as legume as well as oil seed crop and is known for its multiple usages for the society. Almost each plant part is useful such as seeds, which are used for making cooking oil, butter, and confectionary/table preparations, and shoots and oilcakes as cattle feed. Due to diversified use in industry for preparing several delicious preparations including chocolate industry, the importance of this crop has even increased further with reach to every household of the society. This crop also has several other industrial applications such as the use of groundnut oil in preparation for soaps, cosmetics, paint, varnish, furniture polish, lubricating oil, leather dressings, insecticides, and medicine while the use of groundnut shells for manufacturing abrasives, fuel, plastics, wallboard, artificial silk, paper, and glue. This crop also fits into different crop rotations as it increases soil fertility through nitrogen fixation and extend benefits to next crop grown in the same field. It is also an excellent source of nutrition for consumers across the globe and is often served similar to walnuts and almonds.

Groundnut is cultivated over 25.7 million hectares yielding a total 42.3 million tons of produce during the year 2014 with China (42%), India (18%), and Nigeria (7.7%) being the major contributors (http://www.fao.org/faostat/en/#data). Globally, the Asian and African regions together harvest 91% of produce from 95% growing area of groundnut. The last three to four decades could achieve 1.7% yield increase per annum, reaching to the maximum productivity (1823 kg/ha) during the year 2013 (Variath and Janila 2017). Development of improved varieties with high pod yield and resistance/tolerance to biotic and abiotic stresses has played a key role in increasing the productivity of groundnut over the decades. The changing climatic conditions and introduction of groundnut production to new areas have added further challenges to the existing problems of yield reduction due to biotic/abiotic stresses and other unfavorable factors. In addition to agronomic and biotic/abiotic stresses, oil content and oil quality (high oleic acid and low linoleic and palmitic acid) are the two most important traits for genetic improvement as these traits ultimately decide the price of the produce in the market.

The changing climatic conditions and ever-changing consumer preferences demand faster development of improved varieties possessing preferred traits of farmers, consumers, and industry. Genomics has demonstrated great potential in accelerating the process of trait dissection, gene discovery, and molecular breeding.

Therefore, genomics-assisted breeding (GAB) can play an important role in developing improved varieties with desired traits much faster than the conventional breeding (Varshney et al. 2018). In this context, this chapter provides updates on the current status of the germplasm, genetic and genomic resources, and their deployment in trait mapping and molecular breeding in groundnut.

9.2 Germplasm Resources and Priority Traits

India (15,445 accessions), USA (9310 accessions), and China (7837 accessions) have the largest collections of cultivated groundnut germplasm, while Brazil (1220 accessions), USA (1200 accessions), India (477 accessions), and Argentina (472 accessions) have the largest collections of wild groundnuts (see Pandey et al. 2012). These germplasm collections were characterized for various agronomic traits by each GenBank/institute based on the priority of traits required in their breeding program. Since a large number of germplasms cannot be maintained, ICRISAT, USA, and China have developed a core collection with 1704, 831, and 576 genotypes, respectively. Further to reduce the size to make it manageable for breeders, ICRISAT, USA, and China have developed a minicore collection of groundnuts with 184, 112, and 298 genotypes, respectively. Apart from these collections, ICRISAT has also developed two more collections, i.e., composite collection (based on phenotypic data, geographic origin, and taxonomy) and reference set (ICRISAT minicore + other diverse cultivated and wild accessions) (see Pandey et al. 2012). These different germplasm sets provide unlimited opportunities for trait discovery, and their deployment in breeding improved groundnut varieties. Breeders generally work with a limited set of germplasm and show high reluctance in introducing new germplasm to its breeding program due to fear of genetic linkage drag. A systematic evaluation of germplasm in multiple hotspot locations for desired traits and integration of genomics to get rid of linkage drag will provide more confidence to breeders in better utilization of these diverse germplasms in their breeding program.

Trait prioritization is a very dynamic process in groundnut breeding as it differs hugely across different regions, countries, societies, and cultures. In general, each breeding program is engaged in achieving high productivity, high shelling percentage, good pod/seed features, early maturity, resistance to diseases prevalent in target locations, drought tolerance, improved quality, and minimum aflatoxin contamination. The high oil content feature is a very important trait in Asian countries, such as India, where groundnut is used for oil extraction. The early leaf spot (ELS), late leaf spot (LLS), and rust diseases are the universal problem, while nematode and tomato spotted wilt virus (TSWV) are more prevalent in the Americas; bacterial wilt in China, Indonesia, Vietnam, and Uganda; groundnut rosette disease (GRD) and peanut clump virus disease (PCVD) in Africa; and peanut bud necrosis disease (PBND) and peanut stem necrosis disease (PSND) in India (see Variath and Janila 2017). Due to globalization and ease of doing busi-

ness, the industry and consumer-preferred traits, such as aflatoxin contamination, allergens and nutrition, deserve now more attention in the current breeding strategies to enhance research efforts in the coming years.

9.3 Genomic Resources: No More Issue for Genomics and Breeding Applications

There has been tremendous progress in the development of genomic resources for peanut over the last decade, and details of these resources have been reviewed time to time (see Pandey et al. 2012, 2014; Varshney et al. 2013; Ozias-Akins et al. 2017; Vishwakarma et al. 2017a). Six major resources are very useful for peanut research community including (i) reference genome of diploid progenitor species, i.e., *Arachis duranensis* (Bertioli et al. 2016; Chen et al. 2016) and *A. ipaensis* of cultivated groundnut (Bertioli et al. 2016), (ii) high-density genotyping array "Axiom_Arachis" with >58 K highly informative single nucleotide polymorphisms (SNPs) (Pandey et al. 2017a), (iii) gene expression atlas (Clevenger et al. 2016), (iv) genome-wide simple sequence repeat (SSR) and insertion/deletion markers (Zhao et al. 2017; Vishwakarma et al. 2017b), (v) next-generation genetic populations for high-resolution genetic mapping and breeding (see Pandey et al. 2016), and (vi) trait-linked diagnostic markers for use in GAB (see Vishwakarma et al. 2017a).

9.3.1 Reference Genome of Diploid Progenitors and Tetraploid Groundnut

Sequencing reference genome is one of the most important milestones for any crop species for accelerating the process of understanding genome architecture, gene discovery, and molecular breeding (see Varshney et al. 2014b). The International Peanut Genome Initiative (IPGI) initiated the genome sequencing project in 2010 through the Peanut Genome Consortium (PGC) (Wang et al. 2017). This consortium has already completed the sequencing of both the diploid progenitor species, namely, *A. duranensis*V14167 (A-genome) and *A. ipaensis*K30076 (B-genome) (Bertioli et al. 2016), and the finalization of genome assembly for cultivated tetraploid genotype has been completed recently. In addition to IPGI, another initiative, Diploid Progenitor Peanut A-Genome Sequencing Consortium (DPPAGSC), completed the sequencing of another A-genome genotype, PI475845, in the same duration (Chen et al. 2016). The quality of the genome assembly developed by IPGI is much better than the DPPAGSC as the later assembly could not reach up to the pseudomolecule level. The IPGI-led sequencing predicted 1.21 Gb genome size and 36,734 genes for A-genome while 1.51 Gb genome size and 41,840 genes in the B-genome. The DPPAGC-led sequencing predicted comparatively larger genome size (1.05 Gb) and higher

number of genes (50,324) for A-genome assembly as compared to IPGI. The reference genome of these two diploid progenitors are being used in different kinds of analysis such as genome comparisons, mining genome-wide structural variations, transcriptomics, trait mapping, and gene and marker discovery.

Most recently, genome sequencing for the cultivated tetraploid groundnut (cvTifrunner) has also been completed by the International Peanut Genome Initiative (IPGI, https://peanutbase.org/peanut_genome). This initiative has used several modern approaches and technologies for developing a very high-quality genome. This tetraploid genome assembly is 2556 Mbp in size and covers >99% of the actual genome. The scaffold N50, which provides information on assembly contiguity, was reported to be 135.2 MB (IPGI, https://peanutbase.org/peanut_genome). Different sequencing platforms, such as PacBio (48.25×, average read length 11,525 bp) and Illumina (~40×), were used for generating sequencing data. Realizing the allopolyploid genome with very high similarity, this genome was assembled using modern assembling tools including the integration of HiC data/technology. These genome sequences will accelerate the research on trait understanding, genomics, gene discovery, and molecular breeding in groundnut. Further, the availability of resequencing of hundreds of germplasm lines in addition to reference genome will further accelerate the pace of genomics and breeding research in groundnut in the coming years.

9.3.2 High-Density Genotyping "Axiom_Arachis" Array

Among all the structural variations in the plant genome, single nucleotide polymorphisms (SNPs) are the most abundant and hence can be detected very rapidly in large numbers at low cost using next-generation sequencing (NGS) technologies. Initially, genotyping-by-sequencing (GBS) was the most promising approach for generating SNP genotyping data, but high-density SNP array emerged promising too. The GBS platform gives large proportion of missing data which needs further imputation to complete the genetic analysis. The proportion of the missing data is very less or nil in case of SNP arrays, and data can be achieved for all the individuals/markers subjected for genotyping. A high-density SNP array "Axiom_*Arachis*" with 58,233 SNPs was recently developed using the sequencing data of 41 genotypes (30 tetraploids and 11 diploids) (Pandey et al. 2017a). Initially, a large number of genome-wide SNPs (163,782 SNPs) were identified, and then the number of high-quality SNPs were reduced to only 58,233 SNPs for the development of array. In addition to large proportion of SNPs (76.7%) from cultivated groundnut (*A. hypogaea*), this array also had fair representation from other important diploid species *A. duranensis, A. batizocoi*, *A. magna*, *A. stenosperma*, and *A. cardenasii* (Pandey et al. 2017a).

This SNP array has so far been deployed in two studies and has shown great promise in conducting high-resolution genetic studies. For example, Pandey et al. (2017a)

upon deployment of this array in ICRISAT, the "reference set" observed significant loss of genetic diversity in cultivated gene pool and provided greater insights on genetic relatedness and showed preferential selection of genomic regions in the different subspecies of *A. hypogaea*. In another study, Clevenger et al. (2017) genotyped US runner-type breeding material and identified genomic regions with positive selection during the course of the breeding program. This array has provided the groundnut research community for generating high-density genotyping data on genetic and breeding populations for conducting high-resolution trait mapping and more precise breeding.

9.3.3 Gene Expression Atlas

With the availability of reference genome sequence for tetraploid groundnut and its diploid progenitors, now it is a great challenge for the researchers to understand the functions of the entire sets of genes. The gene expression atlas provides detailed information on gene expression in different types of genotypes, organs, tissues, cell, and developmental stages. The information becomes more informative if such data is generated under different stress and treatment conditions. Keeping this in mind, the University of Georgia (UGA), USA, has developed a gene atlas using the RNA sequencing data generated from 22 tissues that represent the critical organs and growth stages. Most importantly, the emphasis was more on the specialized organs and stages such as formation, elongation, and penetration of peg in the soil and then up to seed formation (Clevenger et al. 2016). In addition to identifying 8816 putative homeologous genes, this gene atlas also detected >9000 alternative splicing events and > 6000 noncoding RNAs. Most recently, ICRISAT has also reported development of another gene expression atlas (AhGEA) using RNA-Seq data for 19 tissues from five different stages of an early maturing, high-yielding, drought tolerant groundnut variety, ICGV 91114. This study provided greater insights on understanding the developmental processes and their regulatory network in addition to shedding lights on key biological traits such as seed development, allergens and oil biosynthesis.

9.3.4 Genome-Wide Genetic Markers

The availability of optimum genetic markers is one of the most important milestones in any crop species for use in several genetics and breeding applications. This crop has lacked much-needed resources for genetic analysis and molecular breeding (see Pandey et al. 2012). Nevertheless, the last decade has been very fruitful as the groundnut research community developed a huge number of genetic markers such as simple sequence repeats (SSRs), insertion and deletion (InDel), and single nucleotide polymorphism (SNP) (see Pandey et al. 2016). In contrast to SNPs, the SSR markers are

very useful but are not amenable to high-throughput genotyping. Despite these limitations, SSRs have been well adopted and deployed in most of the crop species. In fact, still a majority of the research groups are using SSR markers especially for use in diversity, trait mapping, and molecular breeding applications in groundnut.

The availability of SSRs has been meager and insufficient in groundnut till the completion of genome sequences for diploid progenitors of cultivated groundnut in 2016 (Bertioli et al. 2016; Chen et al. 2016). Chen et al. (2016) used *A. duranensis* (PI475845) genome assembly and detected 105,003 SSRs and ~8 million SNPs in A-genome. The major problem with these SSRs was that these SSRs cannot be tracked to physical location as the assembly was not up to the pseudomolecule level, thereby restricting the use of these SSRs in genetic studies. Considering this limitation, the high-quality genome assembly developed by IPGI was mined for genome-wide SNPs leading to the detection of large-scale SSRs in both the genomes, i.e., 135,529 SSRs in A-genome (*A. duranensis*) and 199,957 SSRs in B-genome (*A. ipaensis*) (Zhao et al. 2017). For the above-detected SSRs, primers were successfully developed for 112,247 SSRs, i.e., 51,354 (49 SSRs per Mb density) in A subgenome and 60,893 (45 SSRs per Mb density) SSRs in B subgenome, respectively.

The comparative analysis of the draft genome assemblies of both the diploid progenitor species of cultivated tetraploid groundnut identified 515,223 InDels (Vishwakarma et al. 2017a). The sequence comparison of *A. ipaensis* with *A. duranensis* identified 269,973 insertions, while comparison of *A. duranensis* with *A. ipaensis* detected 245,250 deletions. Further, 163,782 SNPs (98,375 SNPs from A subgenome and 65,407 SNPs from B subgenome) were identified upon comparing the whole genome resequencing (WGRS) data of 41 diverse groundnut genotypes (30 tetraploids and 11 diploid accessions) (Pandey et al. 2017a; Clevenger et al. 2017). These genome-wide markers are very useful for conducting genetics and breeding studies in groundnut.

9.3.5 Trait Mapping and Linked Diagnostic Markers

Genomic resources play a key role in genetic dissection and understanding the trait mechanism in any crop species. Further, precise phenotyping data for traits of interest is equally important for genetic analysis and trait mapping. In simple words, the precision and efficiency of trait mapping and candidate gene discovery are directly proportional to the precise trait characterization in diverse genetic materials. The detailed trait mapping efforts and their outcomes have been reviewed time to time (Pandey et al. 2012, 2014, 2016; Varshney et al. 2013; Vishwakarma et al. 2017a; Ozias-Akins et al. 2017), and therefore, this chapter avoids to provide these details again here. Of the several efforts so far using different trait mapping approaches, linked and validated markers are available for only four traits which can be used as diagnostic markers in groundnut breeding. These traits include high oleic acid and resistance to root-knot nematode, rust, and late leaf spot.

Two homologous genes (*ahFAD2A and ahFAD2B*) located on A subgenome (substitution from G:C to A:T) and B subgenome (insertion from A:T) code for enzyme fatty acid desaturase (FAD) that facilitate the conversion of oleic acid to linoleic acid. These two genes have 99% homology, and different types of markers were developed for selecting mutant alleles in breeding program. These markers include allele-specific and cleaved amplified polymorphic sequences (CAPS) markers in addition to SNP-based genotyping (Chu et al. 2009; Chen et al. 2010). Among the diseases causing significant yield in groundnut, the linked markers become available for root-knot nematode caused by a soil-borne pest, *Meloidogyne arenaria*, rust caused by *Puccinia arachidis*, and late leaf spot (LLS) caused by another fungus *Phaeoisariopsis personata*. The DNA-based markers were developed for selecting resistance loci for nematode followed by their validation in US groundnut germplasm (Chu et al. 2007). The genomic regions for resistance to two foliar fungal diseases, rust and LLS, were mapped and linked to SSR markers validated in diverse germplasms (Sujay et al. 2012). Most recently, sequencing-based trait mapping approach, QTLseq, was deployed for developing allele-specific and SNP markers for both the foliar fungal diseases to deploy in breeding (Pandey et al. 2017b). The markers for these four traits have been well standardized in several genomics laboratories across the globe, and their deployment is now a routine in these breeding programs. Most recently, ICRISAT has developed a 10-SNP panel which contains associated SNPs for oil quality (high oleic acid and low palmitic and linoleic acid) and two foliar fungal diseases (rust and LLS). This 10-SNP panel contains associated SNPs for foliar diseases mapped on chromosomes, A02 (LLS) and A03 (rust and LLS) while SNP for high oleic on chromosome B09 (*AhFAD2B*). This panel is now deployed in ICRISAT breeding program for performing early-generation screening using a high-throughput genotyping project (HTPG). This platform, funded by Bill & Melinda Gates Foundation and led by ICRISAT, facilitates genotyping of breeding material at a very less cost, i.e., US$ 1.5 per sample for 10 SNP markers including DNA extraction. Further intensive research is required for developing more diagnostic markers for key traits so that early generation screening can be performed on each seed, before going for planting, to reduce the field and labour resources. Such SNP panels can also be made for ensuring the quality check in seed lots and also checking hybridity in conventional breeding programs.

9.4 Genomics-Based Groundnut Breeding for Achieving Higher Genetic Gains

NGS technologies have not only revolutionized the understanding of genomes and performing high-resolution trait mapping and accelerated gene discovery and marker development but also improved the ease and cost of genotyping with selected markers. The lack of genomic resources has greatly hampered the trait mapping efforts earlier, which got accelerated now with the availability of huge genomic resources. Nevertheless, the efforts with limited resources facilitated development

of linked/diagnostic markers for four important traits in groundnut, namely, high oleic acid and resistance to nematode, rust, and LLS.

US-based groundnut breeding program deployed linked markers for high oleic acid and nematode resistance and improved/developed multiple varieties (Chu et al. 2011). Similarly, ICRISAT deployed linked SSR markers for rust and LLS resistance for improving three popular groundnut varieties (ICGV 91114, JL 24, and TAG 24) in just 3 years' time (Varshney et al. 2014a). This effort not only provided improved lines with enhanced resistance but also showed increased yield and short maturity duration (Janila et al. 2016a, b). ICRISAT also deployed linked markers for achieving desirable proportion of the three key fatty acids (high oleic acid and low palmitic and linoleic acid) in the genetic background of three varieties (ICGV 06110, ICGV 06142, and ICGV 06420) (Janila et al. 2016a). Now the efforts are underway to deploy the linked markers for oil quality and FDR (rust and LLS) for pyramiding in the three popular cultivars (GJG 9, GG 20, and GJGHPS 1). Multiple promising lines developed for foliar disease resistance and oil quality by ICRISAT and its NARS partners in India are currently in national trials for yield assessment and further release. These potential releases (specially high oleic lines) in India will meet the increasing demand of high oleic raw material to domestic and international companies in India.

9.5 Conclusion and Future Prospects

Groundnut is a multipurpose crop with high nutritional value and gained global importance being an important component of the human food basket. Genetic enhancement of groundnut is key to sustain in competition to other crops in terms of key features that drive the demand in the market. NGS technologies coupled with modern genetic and genomic technologies have provided immense hope for achieving accelerated higher genetic gains in less time and resources with high precision and accuracy. Now this crop has ample genomic and genetic resources which were needed to accelerate the process of groundnut improvement. Currently few successful examples of molecular breeding products are available in groundnut; nevertheless, there will be more of such successful stories in the coming years. At the same time, it is also required to test some new breeding technologies and methods such as genomic selection, early-generation screening, and genome editing for getting more precise, faster, and accurate processes to develop next-generation groundnut varieties which can perform better under changing climate conditions.

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Chapter 10
Marker-Assisted Breeding for Economic Traits in Common Bean

James D. Kelly and Nolan Bornowski

Abstract The common bean (*Phaseolus vulgaris* L.) is the most widely grown grain legume species that is consumed directly by humans. The crop is grown from the northern regions of Canada to the temperate regions in the Southern Hemisphere including highland tropical regions of Latin America and East Africa where a wide diversity of seed types and growth habits are produced. Local adaptation is critical, and consumers are very selective in the seed types they grow and consume. Despite its broad adaptation, common bean productivity is low compared to cereal crops and is constrained by a wide range of biotic and abiotic stresses. In addition, grower and consumer preferences for specific growth habits, maturity classes, seed types, and quality traits limit improvement to specific regions and seed types. Breeders are challenged with having to maintain separate breeding programs where outcomes are limited to specific seed types. As a community, many of the biotic stresses are similar, and the genetic tools to control them can be shared. The broad area of marker technologies linked to economic traits is one area where the bean community has shared benefits. This chapter summarizes some of the broad advances in marker technologies as they have been applied to improve economic traits controlling both productivity and quality characteristics of common bean.

Keywords Disease resistance · Dry bean · Molecular markers · Marker-assisted selection · MAS · *Phaseolus vulgaris* · Plant breeding · Quality traits · Quantitative trait loci · QTL · Quality traits

J. D. Kelly (✉) · N. Bornowski
Department of Plant, Soil and Microbial Sciences Michigan State University, East Lansing, MI, USA
e-mail: kellyj@msu.edu; bornowsk@msu.edu

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10.1 Introduction

The common bean (*Phaseolus vulgaris* L.) community has been utilizing marker-assisted breeding (MAB) for the last 25 years (Miklas et al. 2006). Early work started in the 1990s with RAPD markers linked to major disease resistance traits, and the tool was deployed in many labs (Kelly 1995; Kelly and Miklas 1998). To expand their versatility, many of these markers were converted to sequence-characterized amplified region (SCAR) markers, and this further expanded their utility throughout the community. A number of these SCAR markers are still being used in breeding programs decades later (Melotto et al. 1996). In addition, linked markers enabled more detailed fine mapping of major resistance gene loci (Melotto and Kelly 2001). As quantitative trait loci (QTL) analysis tools were developed, work focused on mapping quantitative traits as most traits of economic importance are genetically complex (Kelly et al. 2003; Kelly and Vallejo 2005). The focus of the research expanded beyond disease resistance traits to identify QTL linked to canning quality (Posa-Macalincag et al. 2002; Walters et al. 1997; Wright and Kelly 2011), root traits (Cichy et al. 2009a), drought tolerance (Mukeshimana et al. 2014), insect pest resistance (Brisco et al. 2014), and N-fixation (Heilig et al. 2017). Improved genomic tools finally led to the development of SNP markers and whole genome sequencing of *P. vulgaris* (Schmutz et al. 2014). The development of the BARCBean6K_3 BeadChip with 5389 SNPs (Song et al. 2015) expanded research activities to a whole range of genome-wide association studies (GWAS) focused on an array of agronomic traits (Kamfwa et al. 2015a; Moghaddam et al. 2016) including some related to drought tolerance (Hoyos-Villegas et al. 2016). This work was facilitated by the development of diversity panels, the most notable being the Andean diversity panel assembled by Cichy et al. (2015a). GWAS studies in turn led to candidate gene discoveries for disease resistance traits (Zuiderveen et al. 2016), symbiotic N-fixation (Kamfwa et al. 2015b), and the phytochrome A associated with *ppd* photoperiod gene for flowering on chromosome Pv01 (Kamfwa et al. 2015a). As a community, bean breeders have widely adopted marker-assisted selection despite the absence of detailed genetic maps and the extensive genomic tools available in many of the major grain and oilseed crops. However, the recent genomic sequencing of beans has led to a wide array of genetic studies focused on the origin and domestication, gene synteny within related legume species, identification of resistance gene clusters, and fine mapping of specific economic traits. These studies are discussed in detail below.

Common bean is a diploid member of the *Phaseoleae* tribe with $2n = 22$ chromosomes. The cultivated species are classified into two major horticulture types, one cultivated for dry seeds (commercial bean types typified by pinto or kidney beans; Fig. 10.1) and the other as a vegetable consumed as fresh pods (typified as garden, green, snap, haricot, or string beans). The species *Phaseolus vulgaris* is a self-pollinated annual legume, the most important among the 5 cultivated species with the *Phaseolus* genus that has over 50 recorded species (Freytag and Debouck 2002). Multiple lines of evidence summarized by Mamidi

Fig. 10.1 Diversity in seed size, shape, and color within *Phaseolus vulgaris*. Similar levels of diversity exist for growth habit, performance, and quality traits within common bean

et al. (2013) have shown that common bean is organized in two geographically isolated and genetically distinct gene pools known as Middle American (hereafter referred to as Mesoamerican) and Andean. The wild species was originally considered to be Andean in origin based on rare phaseolin alleles present in wild species found in Peru (Kami et al. 1995). With the advent of genomic research, the actual origin has recently been shown to be in Southern Mexico (Bitocchi et al. 2012). Sequence data of five gene loci support the Mesoamerican origin of the species and the presence of a major bottleneck in the Andean gene pool that predates domestication (Bitocchi et al. 2012). A further study of 102 wild and 112 domesticated bean accessions suggests that domestication occurred independently in the Oaxaca Valley in Mexico, adjacent to the location of maize domestication, and in southern Bolivia and northern Argentina (Bitocchi et al. 2013). Schmutz et al. (2014) estimated that the wild Andean gene pool diverged from the wild Mesoamerican gene pool approximately 165,000 years ago, with a small founding population and a strong bottleneck that lasted approximately 76,000 years. This bottleneck has continued to challenge bean breeders as progress made in breeding in the Mesoamerican gene pool has not been directly transferable to the larger-seeded Andean beans due to lack of genetic diversity (Vandemark et al. 2014).

The bean genome is relatively small, 587 Mb in total across 11 chromosomes named Pv01 to Pv11. Chromosomes were originally numbered by size (Freyre et al.

1998), but a new alignment was made by Pedrosa-Harand et al. (2008) to standardize the nomenclature based on the extensive genetic mapping. The first bean genotype to be sequenced was the Andean landrace, Chaucha Chuga accession number, G19833 from Peru (Schmutz et al. 2014). Aside from opening the floodgates for detailed genetic and genomic studies to proceed, the bean genome sequence described by Schmutz et al. (2014) showed remarkable synteny with the 40 chromosomes of soybean (*Glycine max*). The data support the previous phylogeny of economically important *Phaseoleae* legumes where *P. vulgaris* diverged from soybean some 18 million years ago (MYA) and from cowpea (*Vigna unguiculata*) 4.9 MYA (McClean et al. 2010). The recent publication of the cowpea genome underscores the remarkable synteny between these two species. Six cowpea linkage groups are largely collinear with six common bean chromosomes, while the rest have synteny mainly with two common bean chromosomes (Muñoz-Amatriaín et al. 2016). Based on extensive synteny, the *Vigna* research community has considered renumbering the 11 *Vigna* chromosomes to align directly with those of *P. vulgaris* (Muñoz-Amatriaín, personal communication).

More recently, a second bean genotype BAT 93 from the Mesoamerican gene pool has been sequenced by a multination consortium (Vlasova et al. 2016). Support for the expected gene synteny between the two genomes is displayed by 25,991 protein-coding genes (PCG) mapped in BAT93, where 20,617 were uniquely mapped to 20,618 PCGs in the Andean genome, G19833 (Vlasova et al. 2016). The authors report that 234 of the 852 putative resistance genes identified in the BAT93 genome belonged to the cytoplasmic NBS-LRR class. This compares with 316 NBS-LRR class genes in G19833 that could be mapped to 220 genes in BAT93. However, Vlasova et al. (2016) were unable to find resistance gene clusters that were specific to either of the two genotypes. They suggest that the genomic clustering of resistance genes predates the split of both gene pools and suggest that the differences in pathogen susceptibility might be due to polymorphisms in these loci, rather than a gene presence-absence effect (Vlasova et al. 2016). The full genome sequence of a third bean genotype, OAC-Rex with introgression from the tepary bean (*P. acutifolius*), was recently reported in Canada (Perry et al. 2013). With the decreasing costs for DNA sequencing, a wider array of bean genotypes are expected to be sequenced in the near future and expand the knowledge of the genomic basis of economically important traits of common bean.

10.2 Candidate Gene Clusters

The tagging and mapping of resistance genes in *P. vulgaris* have expanded greatly with the sequencing of the bean genome (Schmutz et al. 2014). The identification of physical positions for individual genes has generated a map with major resistance gene clusters on eight bean chromosomes. Details of the individual clusters are shown in Table 10.1 where genes controlling fungal (*Co*-genes for anthracnose, *Phg*-genes for angular leaf spot, *Ur*-genes for rust), bacterial (*Pse*-genes for halo

Table 10.1 R gene clusters in *Phaseolus vulgaris*

Chromosome	Position (Mb)	Genes	Pathogen	Reference
Pv01	50.16–50.30	*Co-1*	Anthracnose	Zuiderveen et al. (2016)
Pv01	49.81–50.51	*Co-x*	Anthracnose	Richard et al. (2014)
Pv01	ND	*Co-w*	Anthracnose	Geffroy et al. (2008)
Pv01	50.26–50.34	*Co-1^{HY}*	Anthracnose	Chen et al. (2017)
Pv01	50.16–50.55	*Co-Pa*	Anthracnose	De Lima Castro et al. (2017)
Pv01	48.45–50.30	*Co-AC*	Anthracnose	Gilio et al. (2016)
Pv01	50.5	*Co-1^{2}*	Anthracnose	Vazin et al. (2014)
Pv01	50.51	*Co-14*	Anthracnose	Gonçalves-Vidigal et al. (2016)
Pv01	50.51	*Co-1^{4}*	Anthracnose	Gonçalves-Vidigal et al. (2011)
Pv01	50.51	*Phg-1*	ALS	Gonçalves-Vidigal et al. (2011)
Pv01	ND	*Ur-9*	Rust	Kelly et al. (2003)
Pv02	ND	*Co-u*	Anthracnose	Geffroy et al. (2008)
Pv02	40.39–40.44	*Co-u*	Anthracnose	Trabanco et al. 2015
Pv02	48.6	*NN*	Anthracnose	Zuiderveen et al. (2016)
Pv02	48.18–48.27	*Pse-3*	Halo blight	Miklas et al. (2014)
Pv02	48.18–48.27	*I*	BCMV	Bello et al. (2014)
Pv03	ND	*Co-13*	Anthracnose	Lacanallo and Gonçalves-Vidigal (2015)
Pv03	<0.044	*Co-17*	Anthracnose	Trabanco et al. (2015)
Pv03	0.36–0.39	*bc-1^{2}*	BCMV	Meziadi et al. (2016)
Pv03	0.36–0.39	*bc-u*	BCMV	Meziadi et al. (2016)
Pv04	0.59	*Pse-6*	Halo blight	Miklas et al. (2014)
Pv04	ND	*Ur-5*	Rust	Wright et al. (2008)
Pv04	ND	*Ur-D^{a}*	Rust	Miklas et al. (2002)
Pv04	1.23–1.26	*Ur-14*	Rust	Valentini et al. (2017)
Pv04	41.88–45.45	*Phg-4*	ALS	Souza et al. (2016)
Pv04	0.49–0.58	*Phg-3*	ALS	Valentini et al. (2017)
Pv04	0.49–0.58	*Co-3^{4}*	Anthracnose	Valentini et al. (2017)
Pv04	9.08	*Co-15*	Anthracnose	Sousa et al. (2015)
Pv04	1.43	*Co-16*	Anthracnose	Coimbra-Gonçalves et al. (2016)
Pv04	3.36	*Co-3*	Anthracnose	Coimbra-Gonçalves et al. (2016)
Pv04	3.36	*Co-3^{3}*	Anthracnose	Coimbra-Gonçalves et al. (2016)
Pv04	3.36	*Co-10*	Anthracnose	Gonçalves-Vidigal et al. (2013)
Pv04	ND	*Co-y*	Anthracnose	Geffroy et al. 2008
Pv04	ND	*Co-z*	Anthracnose	Geffroy et al. (2008)
Pv07	6.85	*Co-5^{2}*	Anthracnose	Sousa et al. (2014)
Pv07	ND	*Co-6*	Anthracnose	Kelly et al. (2003)
Pv08	2.38–2.48	*Co-4^{2}*	Anthracnose	Oblessuc et al. (2015)
Pv08	ND	*Ur-13*	Rust	Mienie et al. (2005)
Pv08	57.82–58.53	*Phg-2*	ALS	Souza et al. (2016)
Pv10	11.18	*Pse-1*	Halo blight	Miklas et al. (2014)

(continued)

Table 10.1 (continued)

Chromosome	Position (Mb)	Genes	Pathogen	Reference
Pv10	3.49	*Pse-2*	Halo blight	Miklas et al. (2014)
Pv10	39.58	*Pse-4*	Halo blight	Miklas et al. (2014)
Pv10	3.82–8.78	*Phg-5*	ALS	Souza et al. (2016)
Pv11	46.96–47.01	*Ur-3*	Rust	Hurtado-Gonzales et al. (2017)
Pv11	51.93	*Ur-11*	Rust	McClean p.c.
Pv11	ND	*Ur-6*	Rust	Park et al. (2004)
Pv11	ND	*Ur-D*[b]	Rust	Miklas et al. (2002)
Pv11	ND	*Co-2*	Anthracnose	Adam-Blondon et al. (1994)

ND not determined, *NN* not named, *ALS* angular leaf spot, *BCMV* bean common mosaic virus
[a]Ur-Dorado 108
[b]Ur-Dorado 53 (Miklas et al. 2002)

blight), and viral (*I* and *bc*-genes for *bean common mosaic virus*) pathogens co-localize. The resistance gene codes are named to ally with the pathogen name *Co* for *Colletotrichum*. An excellent graphic of these resistance gene clusters in *P. vulgaris* is also provided by Meziadi et al. (2016), and the implications for bean breeders will be discussed further. Aside from the physical mapping, genetic studies have confirmed the absolute co-segregation of gene pairs on Pv01, Pv02, and Pv04. The *Co-1*^{4}and *Phg-1* genes co-segregate at 50.51 Mb on Pv01 (Gonçalves-Vidigal et al. 2011), the *Pse-3* and *I* genes co-segregate at 48.2 Mb on Pv02 (Miklas et al. 2014), and the *Co-3*^{4}and *Phg-3* genes co-segregate at 0.5 Mb on Pv04 (Valentini et al. 2017). Examples of other genes that co-segregate are forthcoming, as additional mapping data is needed to confirm these associations.

Within the *Phaseolus* research community, a genetics committee of the Bean Improvement Cooperative approves all new gene symbols based on genetic and allelism studies and maps positions to ensure that the new gene is unique and provides a real service to breeders worldwide. Identifying alleles is critical so that breeders can choose the most effective allele for a particular pathogenic race or isolate that exists in their region. Not all researchers have chosen to follow the convention, but the physical mapping of genes is now permitting a clearer identification of resistance genes, alleles, and loci that should aid in MAB. All named genes have a number following the symbol that identifies the trait or pathogen, and those without numbers have not received full approval from the genetics committee.

10.3 Chromosome Pv01

The major resistance locus on Pv01 is the Co-1 locus controlling bean anthracnose. Five alleles have been identified at this locus (Ferreira et al. 2013). The *Co-1*^{2} allele has been mapped to a 50.1–50.3 Mb region on Pv01 (Zuiderveen et al. 2016), and Vazin et al. (2014) mapped the *Co-1*^{2} allele to the region of 50.2–50.5 Mb in the cultivar bolt. Three other genes coded *Co-1*^{HY}, *Co-Pa*, and *Co-AC* essentially map to

the same location as the Co-1 locus (Table 10.1). A new gene, *Co-14* in the cultivar Pitanga, was mapped to the same region as the *Co-1*^{4} allele using the same CV 542014 marker (Gonçalves-Vidigal et al. 2016) that Richard et al. (2014) had mapped at 50.51 Mb. Likewise, the *Co-x* gene first described by Geffroy et al. (2008) has been mapped to the same general region (49.8–50.5 Mb) on Pv01 by Richard et al. (2014). It would appear that these genes are either new or existing alleles at the Co-1 locus, but in the absence of thorough allelism studies, breeders are left with no clear choice as to which gene or allele to use. To assist breeders, an InDel marker, NDSU_IND_1_50.2219, was developed that was linked with *Co-1*^{2} at 3.2 cM (Zuiderveen et al. 2016). The InDel marker (50.22 Mb) was present in genotypes possessing different resistant alleles except *Co-1*^{5} (Widusa) at the Co-l locus including the *Co-x* in Jalo EEP558 (Vallejo et al. 2003). The InDel marker should be useful for breeders in third world countries as it can be utilized to integrate the most effective resistance allele at the Co-1 locus using MAB in labs with limited resources. The *Co-1*^{4} allele has been shown to co-segregate with the *Phg-1* gene, which conditions resistance to specific races of angular leaf spot (Gonçalves-Vidigal et al. 2011). Other resistance genes of interest on Pv01 are the *Ur-9* rust avoidance gene from the Andean cultivar Pompadour Checa (Kelly et al. 2003). No physical position has been assigned to this gene, but it is linked to the Bng 122 SSR marker at the distal end of Pv01 (Pedrosa-Harand et al. 2008). Interestingly, all the *Co-1* alleles and the other reported genes including the *Ur-9* gene are found in Andean cultivars, suggesting that this region of Pv01 is strongly Andean in origin.

10.4 Chromosome Pv02

The *I* gene is the most widely deployed and best recognized resistance gene in common bean and resides on the proximal region of Pv02 (Table 10.1). The *I* gene conditions resistance to BCMV through a hypersensitive resistance response to strains of BCMV and was mapped to 48.18–48.27 Mb region of Pv02 (Bello et al. 2014). The *Pse-3* gene that conditions resistance to strains of halo blight co-segregates with the *I* gene (Miklas et al. 2014). The *Co-u* gene first described by Geffroy et al. (2008) was reported to be in close proximity to the *I* gene. Recently Trabanco et al. (2015) mapped *Co-u*to a 40.4 Mb region of Pv02 where prior work had identified a quantitative resistance locus (QRL) for anthracnose resistance in this region (Oblessuc et al. 2014). It is unclear that Trabanco et al. (2015) studied the actual *Co-u*gene as Zuiderveen et al. (2016) identified a genomic region at 48.6 Mb on Pv02 that conditioned resistance to anthracnose in an Andean diversity panel. This region adjacent to the *I* and *Pse-3* genes is a more likely the location of *Co-u* as the SW13 marker linked to the *I* gene (Melotto et al. 1996) co-segregated with the anthracnose resistance gene (not yet named) in a segregating population within the 48.2 to 48.6 Mb interval (Kelly, personal communication). Resistance genes effective against three different pathogens appear to co-localize in this region.

10.5 Chromosome Pv03

Two anthracnose genes and two recessive virus genes are located on Pv03. The *bc-1*^{2}and *bc-u* genes that condition resistance to strains of BCMV are loosely linked (Strausbaugh et al. 1999) and, based on the map of Meziadi et al. (2016), are located around 0.4 Mb in the proximal end of Pv03. However, the SCAR marker SBD5 linked to the *bc-1*^{2} gene allows for direct selection of this allele alone (Miklas et al. 2000b). The *Co-17* gene in the bean accession, SEL1308 that conditions resistance to anthracnose races 3 and 7, was mapped at the extreme proximal end of Pv03 in a region bordered by the InDel marker NDSU_IND_3_00441 at 441 kb (Trabanco et al. 2015). A second anthracnose gene designated *Co-13* and derived from the landrace cultivar Jalo Listras Pretas from Brazil has not been physically mapped (Lacanallo and Gonçalves-Vidigal 2015), but the gene may reside in the same telemorphic region as *Co-17*.

10.6 Chromosome Pv04

This chromosome is home to the largest number of resistance genes and gene clusters. The major Co-3 locus with 5 alleles is located on Pv04: *Co-3* (Mexique1), *Co-3*^{2}(Mexico 227, extinct), *Co-3*^{3} (*Co-9*), *Co-3*^{4} (*Co-10*), *Co-3*^{5} (*Co-7*), in addition the *Co-15*, *Co-16*, *Co-y*, and *Co-x* genes. At least 3 rust-resistant genes, *Ur-5*, *Ur-14*, and *Ur-Dorado* resistant to race 108 (Miklas et al. 2002), the *Pse-6* gene for halo blight, and *Phg-3* gene for angular leaf spot (ALS) that co-segregates with the *Co-3*^{4}allele, are also located on Pv04 (Table 10.1). The majority of the *Co-3* alleles including the *Co-10* gene was tagged with the g2303 marker located at 3.36 Mb (Coimbra-Gonçalves et al. 2016). The same group working with the resistant Ouro Negro cultivar showed co-segregation between the *Phg-3* and *Co-3*^{4} genes, the later formerly named *Co-10* (Gonçalves-Vidigal et al. 2013). These *Phg-3* and *Co-3*^{4} genes were recently mapped to a region 0.49–0.58 Mb (Valentini et al. 2017) on Pv04 quite distant from the original Co-3 locus at 3.36 Mb, so the rationale for renaming *Co-10* gene is unclear. Meziadi et al. (2016) shows a similar clear separation of the *Co-3* and *Co-10* genes based on sequence data of 376 NB-LRR families (see Fig. 10.1). In addition, Souza et al. (2016) previously mapped the *Phg-3* gene with the g2303 marker at 3.36 Mb. A second ALS gene *Phg-4* was mapped to a region 41.88–45.45 Mb toward the distal end of Pv04 (Souza et al. 2016). The *Co-16* gene in Crioulo 159 was mapped at 1.43 Mb (Coimbra-Gonçalves et al. 2016) well within the region the same authors claim that the Co-3 locus resides. A third gene *Co-15* was mapped at 9.1 Mb outside this region suggesting independence from the *Co-3* and *Co-16* genes (Sousa et al. 2015). The *Pse-6* gene also appears to reside within the *Co-3*^{4}*/Phg-3* cluster on Pv04 (Miklas et al. 2014). Two rust resistance genes, *Ur-5* and *Ur-Dorado108* (Miklas et al. 2000a), were never physically mapped, but *Ur-14* from Ouro Negro was mapped to 1.23–1.26 Mb region (Valentini

et al. 2017) adjacent to the *Co-16* gene. That conclusion seems more likely than that drawn by Valentini et al. (2017) where they associate the *Ur-14* gene with the *Co-3*^{4}*/ Phg-3* cluster. Other small pustule rust resistance sources have been mapped to Pv04, but their physical positions were not reported (Wright et al. 2008). The type of resistance reported on Pv04 stands in contrast to the hypersensitive resistance typified by the *Ur-3* and *Ur-11* genes on Pv11. Further work must be done to elucidate the actual number of resistance alleles and resistance genes co-localized at the Co-3 locus.

10.7 Chromosome Pv07

Two anthracnose genes *Co-5* and *Co-6* are located on Pv07. The *Co-5*^{2} allele present in bean accession G2333 was mapped to 6.85 Mb (Sousa et al. 2014) and is clearly distinct from the *Co-6* gene based on numerous genetic studies supporting independent loci (Kelly and Vallejo, 2004). The *Co-6* gene has not been physically mapped, but it appears to localize in a region of Pv07 flanked by the *Phs* (phaseolin) genes at 5.1 Mb and the *P* locus (basic color gene) at 40.47 Mb (Moghaddam et al. 2014).

10.8 Chromosome Pv08

The major Co-4 anthracnose resistance locus resides on Pv08. The *Co-4*^{2} allele is one of the most studied given the broad-based resistance conferred by this allele (Melotto and Kelly 2001; Burt et al. 2015). The complex [*C R Prp*] color locus is linked to the Co-4 locus, which has three known alleles (Kelly and Vallejo 2004). The Co-4 locus occupies a 325 kb region (2.38–2.48 Mb) on Pv08 and contains a unique cluster of 18 *COK-4* genes (Oblessuc et al. 2015). Two other genes have been mapped to the distal end of Pv08 (Meziadi et al. 2016). These include the rust resistance gene *Ur-13* in the cultivar Kranskop (Mienie et al. 2005) and the *Phg-2* gene for angular leaf spot that was mapped to the region of 57.82–58.53 Mb (Souza et al. 2016).

10.9 Chromosome Pv10

Three genes controlling resistant reaction to different races of halo blight were mapped to Pv10 (Miklas et al. 2014). The genes map to different regions of Pv10 suggested independence; however, allelism test has not yet been conducted. The race structure of the pathogen is defined on a set of differential host genotypes and individual resistance genes (*Pse-1, Pse-2, Pse-4*) control either by single or multiple races of the pathogen depending on the breath of resistance spectrum they possess.

For example, *Pse-2* conditions resistance against 7/9 races when tested, whereas *Pse-4* conditions resistance to single race 5 (Miklas et al. 2014). The *Phg-5* gene is also mapped to a region 3.82–8.78 Mb in the vicinity of the *Pse-2* gene. More refined mapping is needed to better localize the *Phg-5* gene.

10.10 Chromosome Pv11

A major cluster of rust resistance genes have been mapped to Pv11. The *Ur-3* and *Ur-11* genes on Pv11 have long been recognized as being linked based on genetic studies using different races of the pathogen, and earlier reports considered them to be allelic differing in number of races they controlled (Stavely 1998). Recent detailed fine mapping of *Ur-3* located the gene at 47 Mb (Hurtado-Gonzales et al. 2017) and *Ur-11* at 51.93 Mb (McClean, personal communication). The close proximity of the two genes underscores the linkage previously reported in traditional genetic studies (Miklas et al. 2002). A KASP marker has been developed for the *Ur-3* gene to facilitate its use in MAB (Hurtado-Gonzales et al. 2017). Other rust-resistant genes, *Ur-6* and *Ur-Dorado* resistant to race 53 (Miklas et al. 2000a), were mapped to Pv11, but no physical position has been reported (Table 10.1). The first anthracnose-resistant gene to be mapped was the *Co-2*gene on Pv11 (Adam-Blondon et al. 1994), and additional linked RAPD markers were reported by Young and Kelly (1996). One of these markers was converted to SCAR marker SQ4 that was also shown to be linked to the *Ur-11* gene (Awale et al. 2008). Further genetic studies placed the *Co-2* in closer proximity to the *Ur-11* than the *Ur-3* gene (Liebenberg et al. 2009) The *Co-2* gene provides resistance to 10 races of *C. lindemuthianum* (Campa et al. 2014) but has lost favor among bean breeders due to a breakdown of resistance to more virulent strains of the pathogen (Kelly and Vallejo 2004), so no further detailed mapping of the Co-2 locus on Pv11 has been pursued.

10.11 Mapping QTL for White Mold Resistance

White mold, caused by *Sclerotinia sclerotiorum*, is one of the most serious production constraints of beans grown in temperate regions of North America and is particularly problematic as more intensive bean production systems are adopted in Brazil (Miklas et al. 2013). Intensive management systems such as higher plant populations, narrow row production, increased fertility rates, and supplemental irrigation can contribute to increased losses from white mold. Genetic resistance is partial, quantitative in nature, and influenced by both the environment and plant growth and architecture traits. QTL mapping for partial resistance has been

underway in beans for almost 20 years, and recently an integrated map of 79 QTL for white mold was generated by Miklas et al. (2013). These QTL can be divided into 27 for resistance, 36 for avoidance, and 16 for root traits associated with lodging resistance. Many of these QTL have no known physical position as mapping was conducted prior to the development of SNP markers for common bean. A recent meta-QTL analysis of resistance to white mold conducted on 37 QTL with a known physical distance condensed that number to 17 loci that included 12 previously identified QTL and 5 new QTL (Vasconcellos et al. 2017). Moreover, nine meta-QTL for resistance were identified and suggested as useful target regions for marker-assisted selection (MAS). In addition to shrinking the number of QTL to a more manageable and useful number, researchers have been able to significantly shrink the interval of two of these meta-QTL, WM7.1 and WM8.3, to improve the precision of MAS efficiency at these loci (Mamidi et al. 2016). Given the important role that plant architecture plays in disease avoidance, Hoyos-Villegas et al. (2015) confirmed that a major QTL WM3.1 was associated with plant architecture avoidance and validated earlier work where RAPD markers were first used to identify this region. Progress in fine mapping these regions will further improve the accuracy of selection for resistance to white mold as phenotypic selection is challenging due to the many controlling environmental factors that influence the development of this disease. Since the same pathogen attacks soybean and given the synteny between beans and soybeans (McClean et al. 2010), future mapping studies and candidate resistance gene identification could be focused on those syntenic regions of common bean where resistance loci have been mapped in soybean.

10.12 Plant Architectural and Photoperiod Traits

Bean plant architecture has been the focus of breeding programs worldwide as the ancestral indeterminate, climbing habit limits production to smaller-scale cropping systems and is not suited to large mechanized farming systems. Four distinct growth habits have been described in beans, and breeding efforts to develop erect upright types suitable for direct harvest were reviewed by Kelly (2001). Type I growth habit is the only determinate bush type, all others are indeterminate differing in length of vine, type II is upright, type III is prostrate, and type IV includes climbing types. A more detailed genetic analysis of upright indeterminate type II genotypes suited for direct harvest revealed that four traits including canopy height, plant length, lodging, and stem diameter are highly correlated and suggests possible anatomical mechanisms responsible for upright plant types (Soltani et al. 2016). A GWAS study of architectural traits in a Middle American diversity panel revealed strong co-localized signals in a narrow genomic interval of Pv07 for three interrelated traits—growth habit, lodging, and canopy height (Moghaddam et al. 2016). The growth habit peak on Pv07 is masked by a stronger peak on Pv01 that is associated

with the *fin* gene present only in determinate plant types. Three terminal flower 1 (TFL-1) homologs associated with determinacy in *P. vulgaris* were previously mapped to Pv01, Pv04, and Pv07. One homolog co-segregated with the *fin* determinacy locus on Pv01 and a second mapped near a second determinacy locus on Pv07 (Kwak et al. 2008). Since the earlier studies were conducted prior to the publication of the bean genome sequence, no physical mapping information is available to associate these regions with the 45.0 to 48.7 Mb region on Pv07 where the main peak for lodging and canopy height is located (Moghaddam et al. 2016). However, the peak identified with determinacy at 45 Mb on Pv01 is near a TFL-1 homolog that maps to the *fin* locus (Repinski et al. 2012). Although determinacy and photoperiod response are interrelated (Kwak et al. 2008), the two traits do not appear to co-localize on Pv01. In a GWAS study of Andean beans grown in a temperate region, Kamfwa et al. (2015a) mapped the days to flower trait at 48.3 Mb on Pv01. This locus appears to be the *ppd* photoperiod gene for flowering and is associated with a phytochrome A candidate gene on Pv01. Candidate genes for flowering, determinacy, and architecture traits were proposed in these GWAS studies but remain to be experimentally validated.

10.13 Quality Traits

Prior to consumption, dry beans must undergo hydrothermal processing to soften the cotyledons and inactivate anti-nutritional factors (Van Der Poel 1990; Martínez-Manrique et al. 2011). As such, dry beans can either be domestically prepared by boiling in water or commercially prepared as a canned product. During canning, beans are soaked and blanched before a brine solution is added, and the cans are thermally sterilized (Matella et al. 2013). Boiling and canning methods both cause physicochemical changes in the beans that influence culinary quality parameters (Wassimi et al. 1990). Quality parameters like water absorption, cooking time, texture, appearance, and color vary among and within market classes (Fig. 10.1) due to both genetic and environmental effects (Hosfield et al. 1984; Hosfield and Uebersax 1990). These quality traits are distinct from agronomic traits and must be selected for accordingly to maintain industry and consumer acceptability. Many studies have focused on physical observations of dry, soaked, or cooked seeds, but fewer studies have been able to relate the actual phenotypic traits with a genetic component. Because culinary traits are typically considered to be under polygenic control, phenotypic selection is largely limited to later generations when most loci are fixed and sufficient seed is available for replicated field trials. The requisite inbreeding and phenotyping are time- and resource-intensive processes that delay germplasm improvement for these traits. Identification of molecular markers linked to quality traits will enable earlier, higher-throughput, and more precise selection. Further advances in biotechnology will facilitate the study of quality traits and their underlying genetic basis.

10.13.1 Water Uptake

Soaking dry beans before thermoprocessing is commonly used to soften the cotyledons and hasten cooking time (Reyes-Moreno et al. 1993). However, there can be significant variation in water absorption due to factors like genotype, soaking solution, and storage conditions (Varriano-Marston and De Omana 1979; Jackson and Varriano-Marston 1981; Elia et al. 1997). A study by Pérez-Vega et al. (2010) was the first study to associate water absorption with genetic markers. Using a recombinant inbred line (RIL) population derived from a Xana (fabada bean market class) x Cornell 49,242 (black bean market class) hybridization, the authors detected two QTL for water absorption, both derived from Xana. The WA3 QTL on Pv03 was found in just one environment and increased water absorption, while the WA4 QTL on Pv04 was found in two environments and decreased water absorption (Pérez-Vega et al. 2010). The WA3 QTL was flanked by markers MCTAETA^{38} and SR20, and the WA4 QTL was flanked by markers SE15 and SH15. Genetic positions of QTL and markers were not given by the authors, but BLAST queries against the *P. vulgaris* genome release V2.1 suggest physical positions for SR20 (Pv03 ≈ 50.18 Mb) and SE15 (Pv04 ≈ 30.6 Mb or ≈39.8 Mb). More recently, Cichy et al. (2014) performed a QTL study on black bean canning quality and found the *Asp* locus on Pv07 conferring shiny seed coat was the closest linked marker to water uptake QTL and best explained the phenotypic variation (R^2 = 48.9%). Other measured traits associated with water uptake (hydration coefficient and washed-drained weight) co-localized around the *Asp* locus. However, while some studies consider the *Asp* locus as a major determinant of water uptake (Bushey et al. 2000, 2001), there may be other factors involved (Brick et al. 2000; Konzen and Tsai 2014). In another study, Cichy et al. (2015b) performed a GWAS using a subset of 206 genotypes from the Andean diversity panel (ADP). Significant SNPs associated with water uptake were found on Pv01, Pv03, Pv06, and Pv07. Notably, the two SNPs on Pv01 (ss715639380 and ss715640804) were the most statistically significant and explained the highest amount of phenotypic variation. Also of interest, the two SNPs on Pv06 (ss715648493 and 715,645,753) explained just 8.3 and 4.6% of the phenotypic variation but had minor allele frequencies of 0.29–0.32. These findings suggest that the markers on Pv01 contribute more to water uptake, but those on Pv06 are better candidates for screening diverse germplasm. Although water uptake is essential for the hydration and cooking of dry beans, there is still limited practical application of MAS for this trait.

10.13.2 Cooking Time

Cooking time is perhaps the most important culinary trait for dry beans on a global scale. The practice of soaking beans to reduce cooking time is not universal, which further exacerbates the time and energy costs involved in cooking beans. While Elia

et al. (1997) determined genotypic differences in cooking times, Jacinto-Hernández et al. (2003) were the first to map cooking time with molecular markers. The authors developed a RIL population from a cross between the quick-cooking Bayo Mecentral (53 ± 15 min) and slow-cooking Bayo Victoria (153 ± 46 min) and evaluated cooking times of the RILs over 3 years. Distribution of cooking times among the progeny suggested that the trait was under oligogenic control. Selected RILs with the shortest and longest cooking times were screened with 14 RAPD markers polymorphic in the parents to find RAPD markers associated with cooking time. Of these markers, 3 were associated with cooking time and were used to screen 70 RILs. A RAPD marker named UNAM 16 was found to be associated with short cooking time when the 310 bp band was present. Due to the high heritability of cooking time and low marker association, the authors recommended against MAS for cooking time. In 2005, Silva and Santos used a combination of RAPD and SSR markers in a bulk segregant analysis study for cooking time. Of the 175 markers, only 1 was found to be associated with the trait. Garcia et al. (2012) used a RIL population derived from a cross between a fast-cooking EMBRAPA line CNFM 7875 and slow-cooking "Laranja" cultivar to identify QTL for cooking time. The most noteworthy QTL, ct1.1, was detected on Pv01 at one location over two generations and explained 20.3% of the phenotypic variation for cooking time. Research by Cichy et al. (2015b) identified several significant SNPs associated with cooking time on Pv02, Pv03, and Pv06. Pv02 contained 2 separate regions at 37.6–38.6 Mb and 48–48.1 Mb that added 17 min and subtracted 7 min from cooking time, respectively. The major allele for the region on Pv03 was responsible for a 21–24 min reduction in cooking time, while the SNPs in linkage disequilibrium on Pv06 were the most significant and increased cooking time by 14.5 min. Interestingly, a neighbor-joining tree analysis featured four of the five fastest-cooking lines on the same branch, suggesting that a similar genetic mechanism is responsible for reduced cooking time in these genotypes. Reducing the cooking time of dry beans will save time, labor, and resources, especially for cooking methods reliant on gathering and burning firewood. These genetic studies provide a foundation for continued research on the potential of marker-assisted selection for cooking time. Although there is general agreement that cooking time is highly heritable and controlled by just a few loci, limited progress has been made in developing useful markers for this trait.

10.13.3 Canning Traits

For many consumers, the convenience of canned beans is preferable to the long preparation time associated with soaking and cooking dry beans. As part of the industrial canning process, beans are cleaned, soaked in a salt solution, quickly heated ("blanched"), and then filled into cans where they are covered in brine or sauce before being heat-sterilized (Matella et al. 2013). These canning conditions induce dramatic physicochemical changes in the seed and may result in beans with undesirable processing characteristics (clumping, viscous broth, nonuniform

soaking/cooking) or sensory characteristics (firm/mushy texture, heavy splitting, color loss) (Wassimi et al. 1990; Hosfield et al. 1995). To meet these industry and consumer standards, dry bean breeders must evaluate elite material for canning quality in addition to yield and agronomic traits. Small-scale canning procedures (Hosfield and Uebersax 1980; Balasubramanian et al. 2000) allow small samples of beans to be processed similarly to commercial canning but require substantial time, labor, and specialized equipment. An alternative to this extensive phenotyping is to identify genetic markers associated with quality traits and implement them in earlier-generation marker-assisted selection. A study by Walters et al. (1997) evaluated three navy bean RIL populations derived from parents contrasting for canning quality. Using RAPD markers, few marker-trait associations were determined due to small population size and lack of polymorphic markers across populations.

Later, Posa-Macalincag et al. (2002) performed a QTL analysis on two kidney bean RIL populations resulting from crosses between acceptable and excellent canning beans. The authors estimated narrow-sense heritability for canned appearance and degree of splitting to be approximately 0.84 and found high correlation between the two traits across environments (r = 91–0.97). The RAPD markers previously identified by Walters et al. (1997) were not polymorphic in these populations, but others were associated with canned appearance and splitting, namely, OP15.1150 on linkage group 1 (putatively located on Pv08) and OG17.1300 on linkage group 2 (unanchored). Wright and Kelly (2011) used a black bean RIL population to map yield and quality traits using SSRs and other markers. QTL were identified for post-processing color retention (7 QTL across 5 linkage groups), canned appearance (Pv05, Pv08), texture (Pv06, Pv11), and washed-drained weight (Pv03, Pv10), although few were detected across multiple years. Most recently, Cichy et al. (2014) used SNP markers to genotype a black bean RIL population derived from crossing shiny black-seeded (*Asp*) and dull-seeded (*asp*) parents. QTL for appearance, color retention, texture, and other quality traits were detected across the genome, though several genomic regions contained clusters of quality traits. Other interesting QTL co-localizations occurred on Pv05 (anthocyanin content, L*, b*, color retention) and Pv11 (L*, a*, b*, color retention, canned appearance). A QTL for canned appearance on Pv08 previously identified by Posa-Macalincag et al. (2002) and Wright and Kelly (2011) was not detected. While MAS has not yet been utilized to improve canning quality traits, the aforementioned studies may be useful in identifying genomic regions for potential marker development.

10.13.4 Post–Harvest Traits

After harvesting, dry beans can be stored for months before being consumed or processed. Seed coat color change resulting from extended storage times, known as post-harvest darkening (PHD), is a phenomenon that is most apparent in pinto, cranberry, small red, pink, carioca, and kidney bean market classes. Consumers perceive

beans with a darker background as too old or harder to cook, which may result in fewer or discounted sales for vendors. Unlike other quality traits, there is a substantial body of research on both physical and genetic mechanisms regarding this trait. Beninger et al. (2005) isolated and quantified polyphenols in a regular-darkening and slow-darkening pinto variety and found that kaempferol levels decreased in the regular darkening bean but not the slow-darkening bean. They suggested that the oxidation of proanthocyanidins may be responsible for seed coat darkening and that a similar reaction may occur in red beans. From a genetic standpoint, at least two loci are involved in PHD: *J* (Prakken 1974; Bassett, 1996) and *sd* (Junk-Knievel et al. 2008; Elsadr et al. 2011).

The J locus, previously recorded as *L* (Schreiber 1940) or *mar* (Lamprecht 1951), causes seed coat after-darkening when the dominant allele is present (Prakken 1970; Bassett 2007). McClean et al. (2002) mapped the J locus to Pv10 and tagged it with the RAPD marker $OL4_{525}$, which they subsequently converted to an STS marker $OL4S_{500}$. Because this marker was developed using Middle American germplasm, it may be gene pool-specific; thus, further marker development is needed for screening Andean germplasm. Recently, Freixas Coutin et al. (2017) used a combined metabolomic and transcriptomic approach to isolate polyphenolic compounds and identify differentially expressed genes between darkening and non-darkening cranberry beans. Regular-darkening beans were found to have greater amounts of proanthocyanidins and increased expression of proanthocyandin biosynthesis genes flavanone 3-hydroxylase 1, dihydroflavonol 4-reductase 1, and anthocyanin reductase 1 *(PvANR1)*, among others. Further research is needed to determine if the relationships between anthocyanin biosynthesis and post-harvest darkening are similar in other market classes. While marker-assisted selection for the J locus is not currently feasible for all gene pools, there is certainly potential for marker development and utilization.

The other locus known to affect PHD, *sd*, confers a slow rate of seed coat darkening when homozygous recessive as described by Junk-Knievel et al. (2008). The researchers crossed slow- and regular-darkening pinto beans, evaluated them by using UV light to artificially darken the seed coats (Junk-Knievel et al. 2007), and analyzed the segregation ratios to decide on a one-gene model. Because the J locus exhibits recessive epistasis over the sd locus, all germplasms must be homozygous for the dominant J allele conferring darkening to discriminate regular- and slow-darkening phenotypes. Felicetti et al. (2012) genotyped slow- and regular-darkening pinto bulks from three segregating F_2 populations to detect SNPs and linked SSRs associated with the sd locus. Three SSRs, Pvsd-1157, Pvsd-1158, and Pvsd-0028, exhibited tight linkage with the sd locus and mapped to Pv07. The authors also demonstrated that these SSRs may be useful to indirectly select for slow-darkening trait in other market classes. These PCR-based markers are now being used in breeding programs for early-generation MAS to develop slow-darkening pinto genotypes.

10.14 Fe and Zn Content

Iron and zinc nutritional deficiencies affect many people around the world, yet high levels of these minerals can be obtained from dry bean seeds. Increasing the content and bioavailability of these micronutrients through biofortification of staple crops like dry beans is a major research focus. Because iron and zinc accumulation are polygenic traits (Ghandilyan et al. 2006), the underlying QTL and genes need to be characterized before marker-assisted selection can be utilized. Using Mesoamerican-derived RILs, Guzmán-Maldonado et al. (2003) mapped several QTL for zinc and iron uptake, but linkage groups were unanchored and of low resolution due to the use of AFLP markers. Blair et al. (2010) genotyped a Mesoamerican-derived RIL population with SSR markers to identify several QTL, including some that co-localized on Pv01 and Pv11. Because dry beans from the Andean gene pool are well represented in global diets, several independent studies have identified QTL for iron and zinc content in Andean germplasm. Cichy et al. (2009b) used a G19833 x AND696-derived RIL population and found co-localizing QTL on Pv01, Pv06, and Pv11, while Blair et al. (2009) used a DOR364 x G19833-derived RIL population to find co-localizing QTL on Pv07 and Pv11. Later, Blair et al. (2011) used a climbing bean RIL population and found an environmentally stable iron concentration QTL on Pv02, an iron concentration QTL near the phaseolin locus on Pv07, and a notable absence of QTL on Pv11 found in other studies. The abundance of co-localizing QTL detected across populations and gene pools suggests similar uptake, or partitioning processes are involved in zinc and iron accumulation, a phenomenon demonstrated in other plants (Frossard et al. 2000; Schroeder et al. 2013). Markers located near large-effect or co-localizing QTL are prime candidates for implementing MAS but need to be validated in the target germplasm beforehand.

Improving iron and zinc content is only one component of biofortification. Divalent cations like iron and zinc are conjugated by anti-nutrients like phytic acid or polyphenols, which lower bioavailability (Frossard et al. 2000). Compared to phytic acid, polyphenolic compounds have a less negative effect on bioavailability. Their effect on iron absorption ranges from moderately inhibitive to negligible (Petry et al. 2015), and their contribution to biochemical process and seed coat appearance may preclude efforts to reduce polyphenolic content for improving bioavailability. Conversely, phytic acid has been shown to have a broad, negative effect on iron and zinc bioavailability (Petry et al. 2015). Plants with low phytic acid tend to have reduced germination, growth, and response to stress (Raboy 2007), yet Campion et al. (2009) identified a dry bean mutant, *lpa1*(280–10), with 90% less phytic acid without the accompanying decrease in agronomic performance. This line was further studied by Panzeri et al. (2011) who identified a recessive mutation in the *PvMRP1* phytic acid transporter gene as a single base pair mutation causing an amino acid substitution. This gene and a homolog, *PvMRP2*, were found to be orthologous to known phytic acid transporters in soybean and rice (Shi et al. 2007). The authors also utilized comparative mapping with soybean (*Glycine max*) to identify linked SSR markers flanking the two genes on Pv01 and Pv07, respectively.

Fileppi et al. (2010) utilized synteny between dry bean and soybean reference genomes to identify and map genes in phytic acid biosynthesis. Extensive synteny with *Arabidopsis* and rice was also observed, reflecting high conservation of genes in this pathway. These studies demonstrate the usefulness of comparative genomics in dry bean research, especially when using the soybean genome as a guide. While genomic information continues to improve, genes involved in iron and zinc accumulation and bioavailability have been already characterized in *Arabidopsis thaliana*, rice (*Oryza sativa*), maize (*Zea mays)*, and soybean (Pilu et al. 2003; Ghandilyan et al. 2006; Xu et al. 2009; Fileppi et al. 2010; Kim and Tai 2011). It is now possible to determine physical positions of orthologous genes in silico as a preliminary step toward marker development, using sequence similarity and genomic resources. Once orthologs are given a physical position in the dry bean genome, they can be compared to published QTL and surrounding markers. MAS is a promising strategy in breeding for nutrition for both increasing micronutrients and minimizing anti-nutrients. Although dry bean breeders may have indirectly selected for enhanced mineral content (McClean et al. 2017), MAS provides additional opportunities for increasing micronutrients and/or decreasing anti-nutrients. The health benefits provided by all grain legumes including common bean are well recognized, but grain legume crops constitute a minor part of most human diets and are greatly underutilized (Foyer et al. 2016). In addition to advantages that grain legumes offer farmers in sustainable crop rotations, N-fixation, and water use efficiency, Foyer et al. (2016) state that "the current lack of coordinated focus on grain legumes has comprised human health, nutritional security and sustainable food production" worldwide.

10.15 Bean Transformation

One area where progress has been more modest is in bean transformation. Problems and progress in bean transformation were reviewed by Veltcheva et al. (2005), and the major challenges and obstacles were discussed by Hnatuszko-Konka et al. (2014). Unlike the progress made in gene transformation in related grain legumes, such as pigeon pea (*Cajanus cajan*; Varshney et al. 2012), chickpea (*Cicer arietinum*), pea (*Pisum sativum*), lentil (*Lens culinaris*), and cowpea (Popelka et al. 2006), only a single successful event has been reported in common bean (Bonfim et al. 2007). The authors used biolistic approach to insert an RNA interference construct to silence the AC1 viral gene and generate transgenic common bean plants that were highly resistant to the whitefly vectored gemini virus, *Bean golden mosaic virus* (BGMV), in Brazil. A single line (Embrapa 5.1) exhibited high levels of resistance upon inoculation at high pressure (300 viruliferous whiteflies per plant) at an early stage of plant development (Bonfim et al. 2007). Crosses and backcrosses were made to commercial carioca cultivars Perola and BRS Pontal, and homozygous near-isogenic lines were developed that exhibited resistance under high BGMV pressure in the field (Faria et al. 2014). This research is an example of the first geminivirus-resistant (bean) plant developed through genetic engineering that has

the potential to impact a large sector of family-based agricultural systems in Brazil (Aragão and Faria 2009). Despite the success, the authors were only able to transform a single genotype, pinto bean cultivar Olathe, and they transferred the transgene through conventional backcrossing into the carioca seed type. Carioca beans are widely grown and consumed in Brazil, and plans only exist to use the RNAi technology in this seed type as GM beans have not been deregulated in any other country except Brazil. Currently, a single elite high-yielding, resistant transgenic line from the backcross program has been released by the Biosafety Network of EMBRAPA after testing over 3 seasons in 31 locations and will represent the first genetically modified common bean cultivar developed in the world (Souza et al. 2018).

10.16 Conclusions

As dry bean research enters the "omics age," adaptation of new technologies and methods will advance the selection methods further. Large-scale sequencing projects have already provided reference genomes, and falling costs are improving access to sequence data for more targeted experiments. First described by Elshire et al. (2011), genotyping by sequencing (GBS) can identify thousands of SNPs that may be used for linkage map construction or aligned to a reference genome if desired. Several GBS protocols have recently been published for dry beans with differing restriction enzymes and experimental objectives (Zou et al. 2014; Hart and Griffiths 2015; Ariani et al. 2016; Schröder et al. 2016). Another potential application of affordable sequencing is genomic selection (GS). Relying on dense marker coverage and prediction models, GS attempts to predict superior genotypes based on genomic estimated breeding values. While MAS is useful for improving large-effect, highly heritable traits, genomic selection may facilitate the improvement of polygenic traits like yield and quality characteristics. However, unlike many small grain crops, dry bean GS may be limited by the need for market-class specific training populations, cost-effectiveness, and lack of prior adaptation. Lastly, the accessibility of sequencing can improve upon current microarray genotyping technology by improving SNP coverage and density across the dry bean genome. Microarray "SNP chips" like the BARCBean6K_3 BeadChip (Song et al. 2015) represent an accessible and convenient genotyping platform but may not contain sufficient marker density or polymorphisms needed for modern genetic studies. Identification of genome-wide, polymorphic, gene-based SNPs through sequencing can create a more robust microarray capable of capturing more genetic variation within and across gene pools and market classes. Dry bean breeders and researchers have a long history of utilizing molecular markers. Applications have ranged from the study of species-wide topics like domestication and disease resistance to market class-dependent quality characteristics. As bioinformatics and sequencing technologies have improved over the past decade, the dry bean community has abundant opportunities to further advance dry bean breeding through MAB.

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Chapter 11
Genomic Approaches to Enhance Stress Tolerance for Productivity Improvements in Pearl Millet

Srikanth Bollam, Vijayalakshmi Pujarula, Rakesh K. Srivastava, and Rajeev Gupta

Abstract Pearl millet [*Pennisetum glaucum* (L.) R. Br.], the sixth most important cereal crop (after rice, wheat, maize, barley, and sorghum), is grown as a grain and stover crop by the small holder farmers in the harshest cropping environments of the arid and semiarid tropical regions of sub-Saharan Africa and South Asia. Millet is grown on ~31 million hectares globally with India in South Asia; Nigeria, Niger, Burkina Faso, and Mali in western and central Africa; and Sudan, Uganda, and Tanzania in Eastern Africa as the major producers. Pearl millet provides food and nutritional security to more than 500 million of the world's poorest and most nutritionally insecure people. Global pearl millet production has increased over the past 15 years, primarily due to availability of improved genetics and adoption of hybrids in India and expanding area under pearl millet production in West Africa. Pearl millet production is challenged by various biotic and abiotic stresses resulting in a significant reduction in yields. The genomics research in pearl millet lagged behind because of multiple reasons in the past. However, in the recent past, several efforts were initiated in genomic research resulting into a generation of large amounts of genomic resources and information including recently published sequence of the reference genome and re-sequencing of almost 1000 lines representing the global diversity. This chapter reviews the advances made in generating the genetic and genomics resources in pearl millet and their interventions in improving the stress tolerance to improve the productivity of this very important climate-smart nutri-cereal.

Keywords Genomics · Markers · Molecular breeding · Nutrition · Pearl millet · Stress tolerance

S. Bollam · V. Pujarula · R. K. Srivastava (✉) · R. Gupta (✉)
International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India
e-mail: r.k.srivastava@cgiar.org; g.rajeev@cgiar.org

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11.1 Introduction

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is one of the principal staple food and earliest used global cereal crops cultivated in the semiarid regions of the sub-Saharan Africa and the central Asian countries. Worldwide about 31 million ha area was covered in 2016 by millet crop with a total production of 28.35 million tonnes (Fig. 11.1). Major pearl millet-producing countries include India, Niger, China, Mali, Nigeria, and Sudan (FAO stat: http://www.fao.org/faostat/en/#search/Millet). India is the largest producer of pearl millet; within the country it occupies 7.14 million ha area under cover with 8.06 million tonnes production with an average productivity of 1132 kg/ha (http://www.aicpmip.res.in/pmnews2017.pdf) and continues to play a critical role in food and livelihood security of millions of people in India (Fig. 11.2). It is the main source of calorie intake for the 500 million people of Asia and Africa. It is a small seeded, highly cross-pollinated, C4 panicoid annual crop

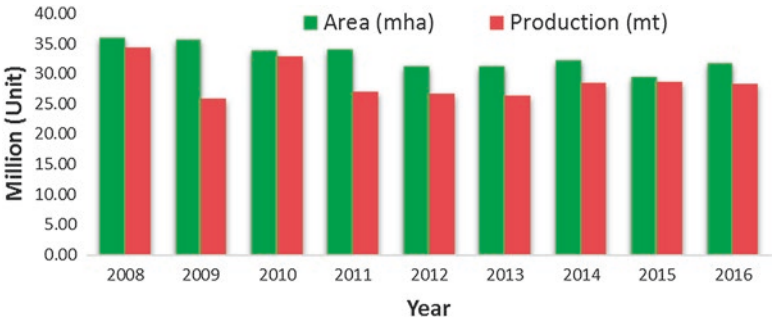

Fig. 11.1 Global millet production and area harvested from the year 2008 to 2016. (Source: FAO Stat)

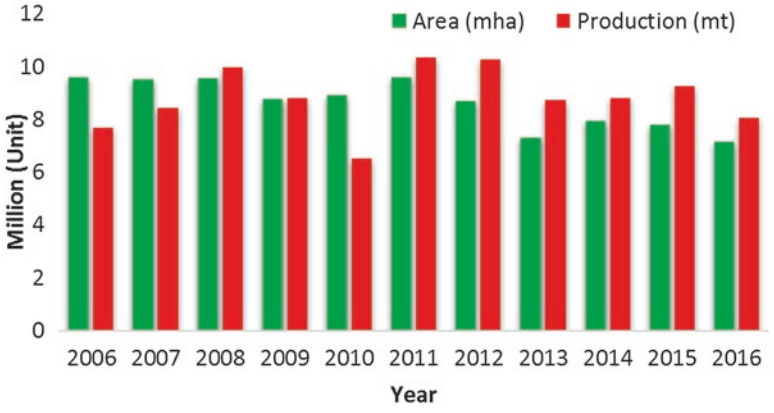

Fig. 11.2 India's pearl millet production and area harvested from the year 2006 to 2016. (Source: AICPMIP, 49th Annual review meeting, 2014 and AICPMIP News letter, 2016 and 2017)

with a genome size of ~2400 Mb and short life cycle (Vadez et al. 2012). Pearl millet is a warm season coarse grain and can be well adapted in the infertile soils with low water-holding capacity, low rainfall, high temperature, and droughty environments, where other cereals would not survive (Vadez 2014; Shivhare and Lata 2016). Apart from its hardy nature to withstand harsh environments, least input demand and outstanding nutritional values make the pearl millet as a highly desirable crop for the arid region farmers. The utility of this crop is diverse, ranging from food to feed, fodder, forage, brewing, biofuel, building material, and fuel for cooking, in dryland areas.

Pearl millet grain has relatively higher energy and contains proteins, vitamin A, B, and carbohydrates; it also contains a higher nutritive value with higher protein (8–60%), carbohydrates (60–78%), fat (3.0–4.6%), and 40% higher in lysine than in feed corn. Pearl millet is a rich source of micronutrients such as iron and zinc with higher quantity than other cereals (Souci et al. 2000). Furthermore, it has higher energy density than sorghum, wheat, and maize (Hill and Hanna 1990). Pearl millet feeding trials in India also confirmed its nutritional values and are superior to maize and rice (DeVries and Toenniessen 2001; Lardy et al. 2004). The biological value of the pearl millet grain protein is higher than wheat, and it is free of gluten and tannin; it contains 5–7% oil, higher protein and energy levels, and more balanced amino acid profile than maize or sorghum (Rai et al. 2008). Apart from human consumption, it is used as animal feed in dairy and poultry industries and alcohol, processed food, and starch industries along with export demand (Basavaraj et al. 2010).

Pearl millet is considered as an important crop species for nutritional security of the poor. The recent advances in tools and technologies and currently available genetic and genomic resources offer immense scope in crop improvement by accelerating the rate of genetic gains. This chapter reviews the recent advances in genomic resources, tools, and technologies and their interventions to address stress tolerance in pearl millet to enhance its yield stability.

11.2 Recent Advances in Genomic and Genetic Resources

In the recent years, whole genome scanning emerged as a potential technology and expanded its utility from cereals to millets. Recently, sequence of reference genome and re-sequencing of 994 pearl millet lines representing the global diversity were accomplished by an international consortium led by ICRISAT (Varshney et al. 2017). In this study, nearly 30 million single nucleotide polymorphisms (SNPs) were identified in pearl millet inbred germplasm association panel (PMiGAP, a panel of 348 lines), in which a total of 450,000 high-quality SNPs were reported after principal component analysis and constructing a neighbor-joining tree. From these released sequenced data, a total of 88,256 SSR markers were identified, in which primers designed for 74,891 SSR markers. These markers will be used for future genetics and breeding application and also help the pearl millet scientists to better understand the trait variation and expedite the genetic improvement of pearl

millet (Varshney et al. 2017). In an earlier study, a total of 83,875 SNPs were identified by using genotyping by sequencing (GBS) in 500 pearl millet lines which have included 252 global accessions and 248 Senegalese landraces (Hu et al. 2015). Moumouni et al. (2015) identified a total of 3321 SNPs in F_2 population of 93 progenies from a wild x cultivated pearl millet cross, out of which 2809 were exhibited high-quality SNPs, a minor allele frequency $\geq$0.3. A total of 314 nonredundant haplotypes for which a single representative SNP marker was used for map construction. These SNPs were evenly distributed over seven linkage groups with an average density of 0.51 SNP/cM. Recently GWAS were performed with 22 SNPs and 3 indel markers in pearl millet inbred germplasm association panel (PMiGAP) comprising 250 inbred lines for drought-tolerant traits (Sehgal et al. 2015). Sehgal et al. (2012) observed the high SNP polymorphism between two parent lines in gene-based DNA sequence in pearl millet. Kumari et al. (2014) developed a set of chromosome segment substitution lines in pearl millet for all the seven linkage groups (LGs). These lines may serve as valuable genetic resources to dissect the genetic effects of complex traits such as yield. Some of the important mapped traits, QTLs and developed markers in pearl millet, were presented in Fig. 11.3, Tables 11.1 and 11.2.

11.3 Genomic Interventions for Abiotic Stress Tolerance

Abiotic stresses pose a serious threat for plant growth and development and causing more than 50% yield reduction in major crops (Bray 2000). Only 10% of the global arable agricultural land fall under non-stress category, and the remaining cultivable lands are prone to abiotic stresses (Dita et al. 2006). The crops affected by abiotic stress were also easily prone to various insects, weeds, and pathogens which in turn add up to the yield losses considerably (Reddy et al. 2004). Pearl millet cultivation is majorly practiced on marginal lands with irregular, untimely rainfall, and environmental stresses; drought stress is the major constraint that ultimately affects the grain yield in low-input farming systems. Besides drought conditions, high temperature and salinity are the other major abiotic stress conditions which affect the pearl millet productivity.

Plant stress response, adaptation, survival, and subsequent yielding in stress conditions are complex mechanisms and are regulated by several cellular, molecular networks along with physiological factors (Ahuja et al. 2010). Therefore, understanding the molecular mechanisms of plant stress tolerance and its adaptation is needed of the hour to overcome the present-changing climatic situations to address the yield stability. Conventional breeding approaches for drought tolerance are more difficult due to location-specific stress factors. Therefore, effective integration of breeding with the support of advanced and cutting-edge "Omics" technologies could be the promising approach to dissect and manipulate the genetic architecture of adaptation to abiotic stresses in crop plants (Langridge and Fleury 2011). In recent times, Omics approaches were extensively utilized in cereals in order to

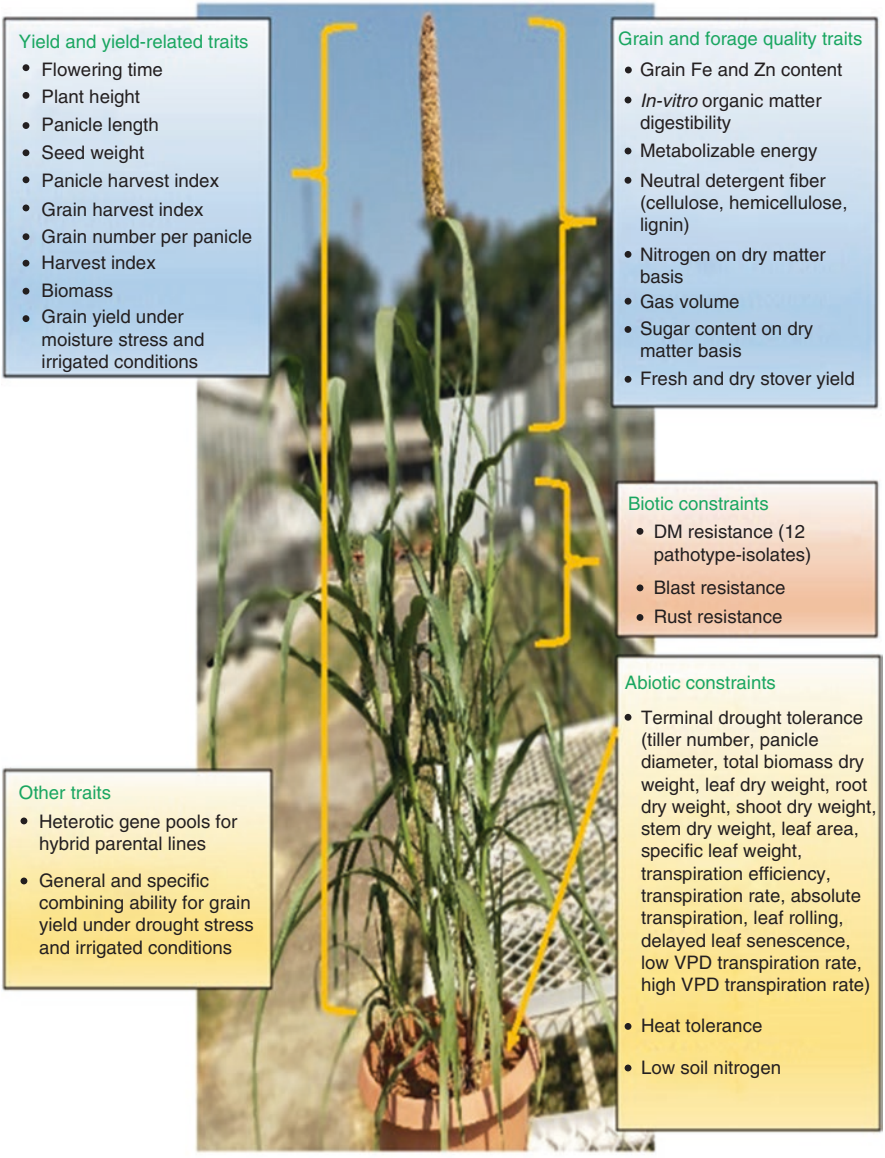

Fig. 11.3 Diagrammatic representation of some of the important traits which are mapped in pearl millet

dissect the genetic architecture of stress adaptation, and quantitative response of abiotic stress tolerance and several QTLs, genes, and genomic loci controlling the adaptive response to harsh environmental conditions was identified, namely, drought adaptive traits (Tuberosa 2012); abscisic acid concentration (Rehman et al. 2011); stay-green (Borrell et al. 2014), canopy temperature traits (Lopes et al. 2014); root

Table 11.1 Some of the important genomic resources and markers associated with different traits in pearl millet

S. no.	Type of markers	Reference
1	450,000 high-quality SNPs, 74,891 SSRs	Varshney et al. (2017)
2	7 SSR markers for iron and zinc	Anuradha et al. (2017)
3	Linkage map constructed with 229DArT and 57 SSR markers developed by genotyping of a RIL population	Ambawat et al. (2016)
4	37 SSRs and CSIP markers, 22 SNPs, and 3 indels for abiotic stresses	Sehgal et al. (2015)
5	745 ESTs in response to drought	Choudhary and Padaria (2015)
6	ISSR-derived SCAR markers for downy mildew	Jogaiah et al. (2014)
7	75 SNPs and CISP developed from available ESTS	Sehgal et al. (2012)
8	100 plus EST-SSRs (developed and mapped in one or more of four pearl millet RIL populations)	Rajaram et al. (2010, 2013)
9	250–280 DArT markers (screened in three pearl millet RIL populations)	Senthilvel et al. (2010)
10	11 finger millet EST-derived SSRs	Arya et al. (2009)
11	4 EST-SSRs and 9 CISPs (polymorphic in populations)	Yadav et al. (2008)
12	21 EST-SSRs and 6 genomic SSRs	Senthilvel et al. (2008)
13	19 EST-derived SSRs	Yadav et al. (2007)
14	16 EST-derived SSRs	Mariac et al. (2006)
15	SSCP-SNP markers by rice and pearl millet EST sequences	Bertin et al. (2005)
16	36 Genomic library-derived SSRs	Qi et al. (2004)
17	18 Genomic library-derived SSRs	Budak et al. (2003) Allouis et al. (2001)

architecture (Lynch et al. 2014); and water-soluble carbohydrate accumulation and its partitioning to storage organs (Rebetzke et al. 2008). Recently, Serba and Yadav (2016) and Shivhare and Lata (2017) have reviewed studies on abiotic and biotic stresses in pearl millet. There is an imminent need for crop plants with improved abiotic stress tolerance to ensure the improved yield stability in pearl millet and thereby contributing to the sustainable dryland agriculture. So far QTLs have been identified, for important traits on different linkage groups were presented in LG1 to LG4 (Fig. 11.4a) and LG5 to LG7 (Fig. 11.4b).

11.3.1 Drought Stress

Abiotic stress, in particular drought stress, causes variations in grain yield loss based on stress intensity, duration, and timing. Among millets, however, foxtail millet, pearl millet, and to lesser extent finger millet have lately started gaining some importance among the research community wherein “omics” have played an

Table 11.2 Quantitative trait loci (QTLs) associated with some of the important traits in pearl millet

S. no.	QTLs and traits	Chromosome	Reference
1	QTLs for agronomic traits	LG1, LG2, LG3, and LG6B	Kumar et al. (2017)
2	QTLs for iron and zinc	LG3	Kumar et al. (2016)
3	QTL for rust resistance	LG1	Ambawat et al. (2016)
4	QTLs for reduced salt uptake	LG2	Sharma et al. (2011, 2014)
5	QTLs for drought tolerance	LG2	Sehgal et al. (2012)
6	QTLs for grain yield in late stress	LG3, LG4, and LG6	Bidinger et al. (2007)
7	QTLs for terminal drought	LG2	Sehgal et al. (2009), Bidinger et al. (2005, 2007), Yadav et al. (2002, 2004)
8	QTLs for grain yield in early stress	LG2 and LG5	Bidinger et al. (2007), Yadav et al. (2004)
9	QTLs for downy mildew	LG1 and LG4	Jones et al. (1995)

important role apart from conventional plant breeding. In pearl millet, significant progress was achieved by using genetic and genomic approaches to identify several genes and their regulatory networks not only to understand the relationships between pearl millet and different cereal crops (Devos and Gale 2000) but also utilized in introgression of QTLs for drought adaptation components (Kholová et al. 2012), terminal drought tolerance, and grain and stover yield (Yadav et al. 2002, 2003, 2004). Several genetic maps for various traits were developed and utilized in breeding of promising QTLs of significant importance (Morgan et al. 1998; Jones et al. 2002; Serraj et al. 2005; Nepolean et al. 2006; Gulia et al. 2007; Bidinger et al. 2007; Yadav et al. 2011).

Drought is the major constraint for pearl millet production in the driest regions of Africa and South Asia. Traditional pearl millet landraces are an excellent source of drought adaptation and able to produce higher grain, biomass, and stover than elite populations (Yadav 2008). Under harsh drought stress conditions, small-panicled and high-tillering landraces produce increased grain yield than landraces with low tillering and large panicles (Van Oosterom et al. 2006). Genotypes with drought tolerance use more water post-anthesis than pre-anthesis, resulting in higher grain yield (Vadez et al. 2013). Post-flowering drought stress is the most critical environmental stress which considerably affects the grain yield and yield stability in pearl millet and leads to crop failure in dryland production systems (Mahalakshmi et al. 1987). Drought stress during grain filling (terminal drought stress) causes severe damage to pearl millet productivity than vegetative stage stress, as pearl millet's rapid growth and asynchronous tillering allow the plant to recover from vegetative stage drought stress but fails in the case of terminal drought stress (Bidinger et al. 1987). In pearl millet under terminal drought stress conditions, significant

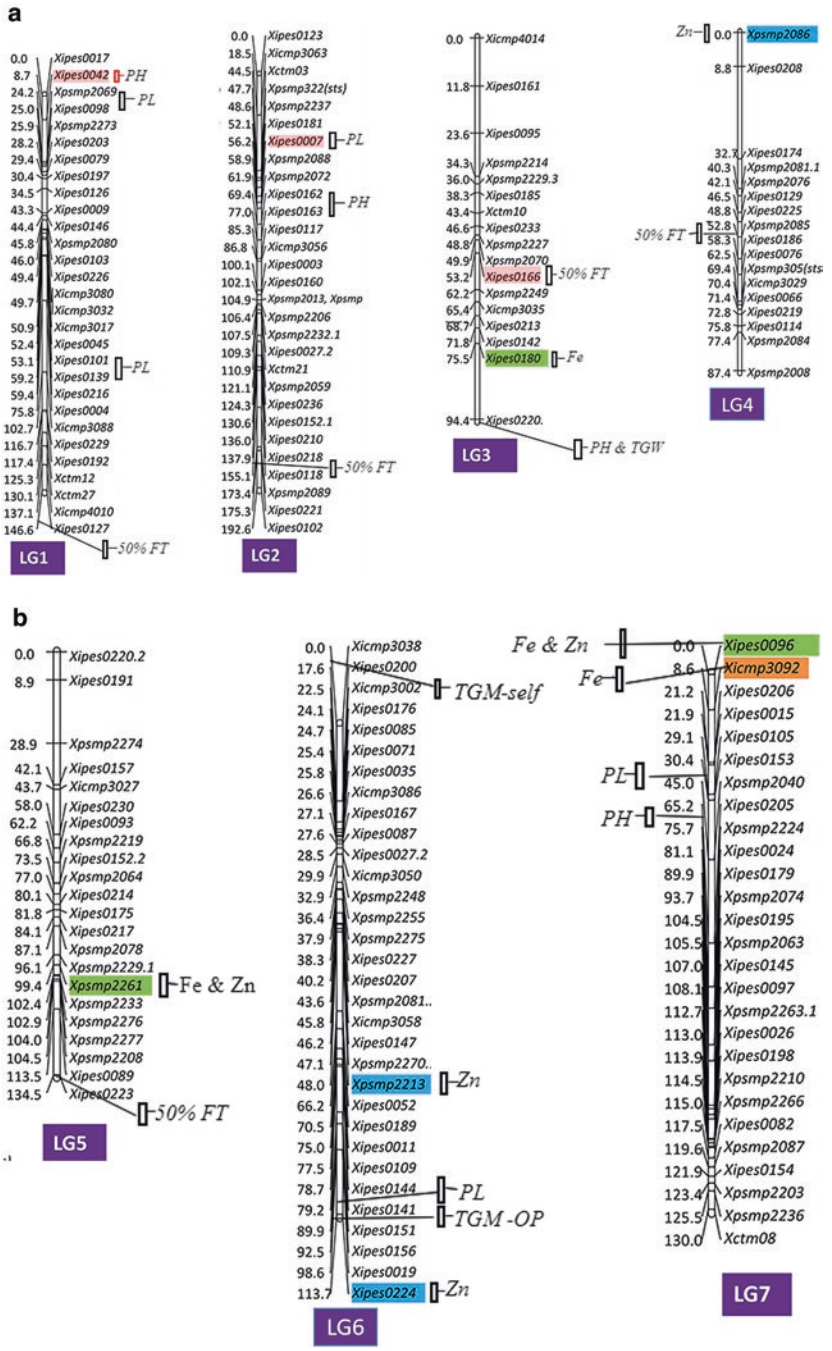

Fig. 11.4 (**a**, **b**) Consensus map with QTL positions for important traits. (Source of linkage map, Anuradha et al. 2017). FT, time to 50% flowering (d); PH, plant height (cm); PL, panicle length (cm); TGW_self, self-pollinated 1000-grain weight (g); TGW_OP, open-pollinated 1000-grain weight (g); Fe, grain iron; Zn, zinc

progress was made in mapping QTLs for components of stover and grain yield, along with yield stability using progeny of two sets of mapping populations, namely, H 77/833–2 and PRLT 2/89–33 and the other from a cross between elite inbred parents (ICMB 841 and 863B) (Yadav et al. 2002, 2003, 2004; Hash et al. 2003; Bidinger et al. 2007). These two crosses identified a major QTL and confer to terminal drought tolerance on linkage group 2 (LG2) with 23% of the variation explained (PVE). In the same LG2 region, QTLs for biomass and harvest index were also colocated which suggest that increased terminal drought tolerance might enhance the dry matter partitioning to the grain (Yadav et al. 2002) due to this genomic segment. Kholova et al. (2011) studied the physiological (transpiration efficiency and ABA concentration) and biochemical (contents of the photosynthetic pigments and reactive oxygen species (ROS) enzyme activities) activities in drought-sensitive and drought-insensitive pearl millet lines and QTL NILs for dissecting the drought QTL and concluded that the pigment content and ROS machinery are not playing their vital role in pearl millet terminal drought stress. It was further concluded that the DT-QTL on LG2 is crucial for constitutive water storage mechanism and helps the plant at the time of grain filling during terminal drought stress (Kholova et al. 2011). The positive effect of this QTL on yield and other yield components under saline and alkaline conditions was also tested and confirmed (Sharma et al. 2014). Recently, multiple efforts are actively going on to introgress these important QTLs into elite lines using marker-assisted selection (MAS) to develop drought-tolerant pearl millet (Srivastava, Gupta et al. unpublished).

11.3.2 Nitrogen Use Efficiency (NUE)

The global demand for nitrogen (N) fertilizer for agricultural production, which already stands at ~110 million metric tonnes per year, is projected to increase to ~250 million metric tonnes by the year 2050 (FAO.org; Frink et al. 1999; Good et al. 2004; Tilman 1999; Tilman et al. 2011). India is the second largest producer and consumer of chemical fertilizers in the world (Abrol et al. 2017). Globally about 50% of human population relies on N fertilizer for food production (Smil 2001). Nitrogen (N) is the major fertilizer input, and it is the key nutrient element for crop productivity. For the complete realization of the higher yield potential, enhanced fertilizer N application is compulsory (Roberts et al. 2009). It is estimated that >40% of the human-consumed protein-nitrogen was derived from nitrogen fertilizer (Smil 2001). About 60% of the world's N fertilizer is used for producing three major cereals, namely, rice, wheat, and maize (Ladha et al. 2005). Because nitrate is very mobile in the soil, substantial amount (>50% in some cases) of applied N is lost by leaching, runoff and denitrification. In addition to increase in cost of crop production, in the long run these processes of N loss (Raun and Johnson 1999; Good et al. 2004; Hakeem et al. 2011; Prasad 2013) not only pollute the ground water and adversely affect soil structure but also have detrimental effects on environment such as increase in nitric oxide, ozone, etc. (Ramos 1996). Hence, developing crop varieties with improved efficiency for N absorption and utilization will help mitigate

these problems to some extent (Frink et al. 1999; Good et al. 2004). Nitrogen is an essential component of cellular physiology and present in several compounds including nucleoside phosphates and amino acids which are basic building blocks of nucleic acids and proteins, respectively. In general, N most often limits plant growth and agricultural productivity because naturally soil particles do not contain many N-containing minerals, and in addition N can be readily lost from the rooting environment (Duvick 2005). Most of the plant roots absorb N from the soil in the form of nitrate, which, aside from being assimilated to a minor extent into amino acids in the root, is transported to the leaf for reduction and assimilation (Crawford and Glass 1998). Nitrate is reduced to nitrite by nitrate reductase (NR) in cytosol, whereas nitrite is further reduced to ammonium by nitrite reductase (NiR) in chloroplast, and ammonium is then assimilated into amino acids or other compounds (Buchanan et al. 2000). Influx of nitrate into the root cells is accompanied by efflux, which is favored because the interior of the cell is negatively charged, and nitrate, being an anion, is taken up against an electrochemical gradient. Efflux goes up significantly as the concentration of nitrate around the root surface increases, accounting for up to 30% of the total nitrate absorbed (Volk 1997). Absorption and reduction of nitrate into ammonium are highly energy-intensive process suggesting that crops with improved NUE will save lot of energy thus the biomass and yield. Improving nitrogen use efficiency (NUE) in crops is one of the major initiatives for both private and public researchers because the outcome will not only increase the profit margins for the farmers by reducing the input costs but also save the environment from degradation by reduced use of chemical fertilizers.

Being its complex nature, NUE can be influenced by both internal and external factors. In India, the current average NUE in the field is approximately 33%. Through efficient N management practices, NUE can be improved if the cultivar is responsive. Thus, developing high NUE genotypes becomes the major objective for pearl millet breeding programs in the current agricultural scenario. High NUE cultivars can be defined by their ability to produce higher grain yields under low N inputs (Ladha et al. 1998). The core idea is to get the maximum possible yield with optimal nitrogen inputs which can compensate the compromised yield with economic and environmental benefits.

With the priority of low nitrogen input and sustainable and environment-friendly agriculture, NUE of pearl millet becomes very important in modern agriculture. However, increase in global yield was not kept in pace with the excessive fertilizer usage and also subsequent increase of the cost of cultivation and immediate negative environmental impacts. The primary goal of utilizing nitrogen fertilizers is to increase in yield per a unit land area, for this achievement fertilizer nitrogen usage must be within the optimum and economic level (Firbank 2005). However, the fertilizer demand of high yielding varieties and hybrids was high; N fixation and organic nitrogen recycling were not in line with the food requirements of the growing global population. In the past few years, conventional breeding practices improved the crop productivity as well as the NUE. Thus, to minimize the loss of nitrogen, a comprehensive approach to reduce or optimize nitrogen usage, improvement of NUE for genotypes, and their N management strategies were urgently needed. Several critical reviews elaborated the challenges associated with excess fertilizer nitrogen usage

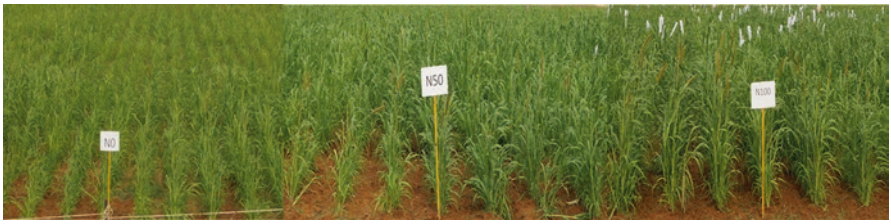

Fig. 11.5 Differential N response of pearl millet lines under field condition at ICRISAT during summer season 2018

and the need for developing the NUE (Pathak et al. 2008; Garnett 2009). It was proposed that annual increase of 1% NUE could save $1.1 billion (Meena et al. 2016). Therefore, it is important to develop and cultivate the crop varieties with higher NUE for reducing nitrogen loss to the environment for sustainable agriculture. Several workers have made attempts to study the genetic differences of nitrogen uptake and yield per unit nitrogen application in crop plants such as rice, wheat, maize, sorghum, and barley (Ortiz-Monasterio et al. 1997; Le Gouis et al. 2000; Presterl et al. 2003; Anbessa et al. 2009; Namai et al. 2009). In pearl millet systematic attempts are in progress to understand the regulatory mechanisms, which control nitrogen usage and metabolism, particularly in N-limited conditions. As part of "Cambridge-India Network for Translational Research in Nitrogen" (CINTRIN) project, scientists from ICRISAT are trying to identify nitrogen use efficient lines under low and high N conditions for the marginal and favorable ecologies, respectively. A total of 400 pearl millet cultivars including the association mapping panel (PMiGAP) and parents of mapping populations were grown in the precision field of ICRISAT at three (0%, 50%, and 100% of the recommended nitrogen doses) nitrogen levels in alpha lattice design with two replications during multiple seasons in the recent years (Fig. 11.5). The trials were evaluated for different morphological (five traits), physiological (five traits), agronomic (five traits), and yield characters (six traits). In two seasons, wide variations were observed among the measured traits. Irrespective of genotypes, in all measured traits, the highest grand mean was observed in N100, followed by N50 and N0. A few genotypes were shown good performance in minimum fertilizer condition. Data compilation and analyses are under way (CINTRIN, unpublished data). Some of the genotypes showing better chlorophyll content in all the three treatments, namely, IP16120, Jakhranas 8–35-2-P2, H77/833–2-P5, W504–1-1, and IP3201. Few mapping population parents, including Jakharana S8–35-2-P2/RIB 334/74-P1, are showing substantial differences in terms of chlorophyll content under N-0 conditions during summer 2017. These mapping populations seem promising for the mining of QTL/gene/genomic regions associated with chlorophyll content and can be effectively used for mapping studies (CINTRIN, unpublished data). Data obtained from the 400 pearl millet cultivars from the two seasons will be utilized for genome-wide association studies (GWAS). Based on the genotypic performance under three nitrogen levels, nitrogen-sensitive (NS) and nitrogen-insensitive (NIS) pearl millet genotypes will be used for transcriptome studies to identify nitrogen use efficiency genes in pearl millet.

11.3.3 Salinity Stress

Salinity is one of the major abiotic stresses in arid region crops where water evaporation is high and makes it unavailable to the plants. Salinity stress is severe in the regions affected by extreme drought and high-temperature stress which increase the capillary water to the upside and the soluble salts to the root zone (Várallyay 1994). Pearl millet is cultivated in less fertile soils with a minimum amount of organic matter and low levels of phosphorus (Yadav and Rai 2013). Pearl millet is a hardy plant that can survive in the saline lands for forage and grain production, though it can be considered as significant abiotic stress in the west and north zone of central Asia. Only limited information is known about salinity stress in pearl millet; there is an imminent need to understand the molecular and physiological mechanisms in order to develop the salinity tolerance in pearl millet. In pearl millet reduction in shoot nitrogen content and increase in K+ and Na + content are associated with salinity tolerance (Dwivedi et al. 2012). At vegetative stage, shoot biomass ratio and Na+ contents were considered as potential traits for screening salinity tolerance in pearl millet (Krishnamurthy et al. 2007).

11.3.4 High–Temperature Stress

In pearl millet, high-temperature stresses at both seedling and reproductive stages have a significant impact on crop establishment and its productivity. Pearl millet is well adapted to the arid regions, and most of the growth stages such as seed germination, photosynthesis, and coleoptile elongation require 35 °C (Garcia-Huidobro et al. 1985). The temperature above 35 °C could be harmful to the development of major cereals, but pearl millet could sustain growth as well as yield potential in the hot environmental conditions. It was reported that pearl millet seed germination occurs at 35–45 °C, further increase in temperature to 47 °C it decreases and more or less stops at 50 °C (Garcia-Huidobro et al. 1982). Increase in the temperature of seedbed causes poor plant stand which ultimately affects yield. It was reported that the pearl millet seedlings of the first 10 days of sowing are more vulnerable to temperature stress (Stomph 1990). Pearl millet breeding line H77/833–2 is tolerant to high-temperature stress and sensitive to drought; hybrids of it are extensively used in northwestern region of India (Yadav et al. 2014). Mapping population developed by this cultivar was used to map terminal drought tolerance, grain yield, and stover yield and yield component QTLs in pearl millet (Nepolean et al. 2006). Recently, it was reported that there is genetic variation for heat tolerance at seedling and reproductive stage among germplasm; the reproductive stage heat-tolerant breeding line, IP 19877, has shown same seed set as that of 9444, a tolerant check (Gupta et al. 2015). Multiple efforts are underway at ICRISAT to dissect heat tolerance trait in pearl millet at genetic and molecular levels.

11.4 Genomic Interventions for Biotic Stress Tolerance in Pearl Millet

Like any other crops, pearl millet is also susceptible to several biotic stresses. Major biotic stresses include fungal infections such as downy mildew, blast, rust, ergot, and smut which impact the pearl millet production and grain yield.

11.4.1 Downy Mildew (DM)

DM is the most devastating yield constraint for pearl millet production, caused by *Sclerospora graminicola*, an obligate biotrophic pathogen, which results in exhaustive yield loss up to 80% (Singh et al. 1993; Howarth and Yadav 2002). DM majorly attacks pearl millet panicles, and its effect ranged from minor symptoms to disasters when major fields have been destroyed. Moderate temperature (20–30 °C) and high relative humidity (85–90%) favor the DM disease incidence, and it is clearly identified by leaf chlorosis, leafy inflorescence, and seed set failure (Thakur et al. 2008). This disease was first reported in India and was considered a minor impact of pearl millet yield production until 1970. However, in 1970–1971 estimated annual grain yield of popular Indian pearl millet hybrid (HB3) is approximately 8.2 million metric tonnes (Singh 1995). In the following year (1971–1972), severe yield loss of HB3 has been observed to about 4.6 million metric tonnes due to epidemic of DM (Dwivedi et al. 2012). So far, different techniques and resources have been developed for the identification and screening of virulent traits for DM at different ICRISAT centers. Further, effective phenotypic methods are also developed for DM field screen (Jones et al. 2002; Thakur et al. 2008). Recently Siddaiah et al. (2017) published the draft genome sequence of pathotype 1, which is one of the most virulent pathotypes of *S. graminicola* from India. This sequence information will be useful for breeding program to develop DM resistance varieties. Development and commercialization of new hybrid cultivars have been increased in the last 10 years, which give raises to new strains of DM pathogens. Almost 10 years back, in India six major DM pathotypes have been reported (Thakur et al. 2006). However, recently Thakur et al. (2011) reported about 20 virulent pathotypes of *S. graminicola* suggesting that in the coming years, new more virulent strains could be identified. The inheritance of DM resistance is a quantitative character. DM resistance shows dominance over susceptibility and recessive traits, though part of host plant resistance is governed by one or several genes along with modifiers (Hash and Witcombe 2001; Breese et al. 2002; Dwivedi et al. 2012). DM resistance QTLs have been mapped by screening *S. graminicola* pathogen and F4 mapping populations (from India, Nigeria, Niger, and Senegal), namely, LGD-1-B-10 x Blast ICMP 85410UK and 7042(S)-1 x and P 7–3 by field and greenhouse screening in India and the UK; it was also used to test the efficiency of greenhouse screen resistance indicators in field conditions. Two DM resistance QTLs, which are consistent, were

detected on LG1 and LG2 in field and glasshouse screens of the UK and India; however, LG1 QTL is showing up to 60% higher variation than LG2 QTL (up to 16%) (Jones et al. 1995, 2002). Number of loci which contributed about 17.4% and 47.7% of the total inheritance of the resistance to DM incidence and severity in pearl millet was reported (Angarawai et al. 2009). Jones et al. (1995) mapped a major DM resistance QTL detected on LG1 from India, against the Nigeria and Niger pathogen populations on LG4, and also on LG2, against Senegal pathogen population. Interestingly there was no QTL effective against all the four pathogen populations, suggesting that pathotype-specific resistance is a major mechanism of DM resistance in this cross (Jones et al. 1995). Liu et al. (1994) used F2 population plants to construct the map using RFLP markers. Several other workers used RFLP markers for DM resistance QTL mapping on these LGs (Breese et al. 2002; Gulia et al. 2007). SCAR marker was developed from inter-simple sequence repeats associated with DM resistance linkage group in pearl millet (Jogaiah et al. 2014). Hash et al. (2006) reported that gene pyramiding could be the promising approach to enhance the resistance toward diverse isolates of DM; in his study, pearl millet inbred lines (48) were studied against nine different *S. graminicola* isolates from five geographical regions across India to confirm the hypothesis. Developing DM resistance varieties has become the highest priority for pearl millet breeders. Resistance to DM has been reported in some pearl millet germplasm accessions and hybrids. Singh (1995) reported that ICMH451 and Pusa 23 are popular resistant hybrids; likewise, a top cross hybrid ICMH 88088 was developed by ICRISAT, highly DM resistant and also producing high yield. In India, four widely cultivated OPVs such as WC-C75, ICMS7703, ICTP8203, and ICMV155 are resistant to DM (Shivhare and Lata 2017). ICRISAT also developed a few DM resistance varieties for Western Africa, namely, ICMV1 and ICMV2 for Senegal and IKMP2, IKMP3, and IKMV 8201 for Burkina Faso (Singh et al. 1993). Development and commercial deployment of downy mildew-resistant version of HHB 67, a popular hybrid being grown in North India, are the first successful story of marker-assisted breeding (MAB) in field crops in public domain in India (Hash et al. 2006). A number of quantitative trait loci (QTLs) for downy mildew resistance have been identified on different linkage groups, and some of them are specific to different pathotypes (Hash and Witcombe 2001; Jones et al. 2002). DNA markers have been identified for about 60 different putative DM resistance QTLs in pearl millet (Breese et al. 2002; Hash and Witcombe 2002). A number of downy mildew-resistant QTLs effective against diverse Indian pathotypes of *S. graminicola* have been transferred to the commercial B-lines (843B, 81B) and R-lines (H 77/833–2, ICMP 451). In a recent study, Jogaiah et al. (2016) suggested the potential of the biocontrol agents to resist DM in pearl millet. A recent study using transcriptome analysis of DM-resistant and susceptible genotypes upon infection and control using NGS platform reported that, in the resistant genotype, upregulation of pathways for secondary metabolism especially phenylpropanoid pathway was observed. Upregulation of defense responsive transcripts, namely, R genes, HR-induced proteins, PR proteins, and plant hormonal signaling transduction proteins were also observed. In resistant genotype, the

Fig. 11.6 Downy mildew resistance and high grain iron (Fe) and zinc (Zn) content double QTL introgression hybrid trial at Bikaner, India, during rainy season (2017)

transcripts for V-type proton ATPase, purothionin, and skp1 proteins showed highest expression. This study also suggested the potentials of systemic acquired resistance and hypersensitive response as possible machinery operating the defense mechanism in pearl millet DM resistance (Kulkarni et al. 2016). During rainy season (2017), hybrid trial has shown high level of downy mildew resistance by introgression of double QTL (Fig. 11.6).

11.4.2 Rust

Rust is one of the major constraints for pearl millet production across the world and not only causes up to 76% yield loss but also affects fodder yield and its quality (Wilson et al. 1996). This disease was first reported on pearl millet in India during 1904 as being caused by *Puccinia substriata var. indica* (Wells et al. 1973). Effect of rust can be austere ranging from death of the affected young plant from early infection to desiccation or death of leaves with later infection. Several studies suggested that green yield, dry matter, and in vitro digestibility are negatively correlated with the rust incidence (Monson et al. 1986; Wilson et al. 1991, 1996). Several attempts have been made to identify the markers/ QTLs controlling rust resistance in pearl millet. It was reported that rust resistance controlled by a single dominant gene individually named as Rpp1, Rr1, Rr2, and Rr3, respectively, and rust susceptibility controlled by its recessive allele, with numerous diverse sources of the major gene and its quantitative resistance have been extensively studied, identified,

and exploited (Hanna et al. 1985; Singh and Singh 1987; Singh 1990; Wilson 1993, 1994; Wilson et al. 2001, 2006). A combination of RFLP and RAPD markers was used to map the Rr1 gene on LG3 in wild pearl millet (Morgan et al. 1998) though gene resistance was overcome by the pathogen in the southeast USA soon after its introgression in popular grain and forage hybrids followed by its backcross with a hybrid parent maintainer background Tift 85D2A1/85D2B1 (Hanna et al. 1987; Wilson 1993, 1994). Three segregating populations were screened with RAPD (random decamer) primers and RFLPs using a core set of single-copy markers (probes detected) on the pearl millet genetic map (Liu et al. 1994). Similarly, Morgan et al. (1998) also identified rest resistance genes in three segregating populations with RFLP and RAPD markers and resulted in *Rr1* rust resistance gene from the pearl millet subspecies *P. glaucum* ssp. *monodii* which was linked 8.5 cM from the RAPD OP-8350. In the same study, genetic linkage map was constructed with RFLP markers in both Tift 89D2 and ICMP 83506, while rust resistance genes were located linkage group 4. The linkage of two RFLP markers, Xpsm108 (15.5 cM) and Xpsm174 (17.7 cM), placed the Rr1 gene on linkage group 3 of the pearl millet map. Another RFLP marker Xpsm716 closely linked to rust resistance gene in ICMP 83506 effective against race PS92–1, but the gene was not found effective against Patancheru isolate of *P. substriata var. indica* in India (Sharma et al. 2009). In a recent study, an integrated high-density genetic linkage map was used to map the QTLs for rust in pearl millet by using DArT and SSR markers in a RIL population comprising of 167 F7 plants which are segregating for rust resistance developed from a cross between 81B-P6 (susceptible) and 9 ICMP 451-P8 (resistant). Out of the 167 RILs, 32 lines were resistant, 18 moderately resistant, 73 moderately susceptible, and 40 susceptible, and the remaining 4 lines were highly susceptible to rust. A major QTL for rust with 58% phenotypic variation was mapped on linkage group 1 with LD score of 27, and two small effect putative modifiers were also detected on LG4 and LG7 explaining 9 and 8.3% of the observed phenotypic variance. It was also reported that the novel rust resistance QTL which was identified on LG1 is also associated with the slow-rusting phenotype of importance (Ambawat et al. 2016). Few rust-resistant varieties have been reported in some pearl millet germplasm accessions and breeding lines (Wilson 1993; Singh et al. 1997). However, lines that were resistant in India became susceptible in the USA indicating the existence of different physiological races in India and the USA (Wilson et al. 1991; Tapsoba and Wilson 1996). Sharma et al. (2009) identified the four resistance lines including one B-line (ICMB 96222) and three R-lines (ICMR 0699, ICMP 451-P8, and ICMP 451-P6), and other four R-lines were susceptible to rest.

11.4.3 Blast

Blast or *Pyricularia* leaf spot is the second most serious fungal foliar disease of pearl millet in India and the USA causing substantial yield and forage losses. This disease is caused by *Pyricularia grisea*. Symptoms include grayish, water-soaked foliar lesions that further enlarge and become necrotic, causing extensive chlorosis and premature drying of young leaves (Wilson et al. 1989). Blast disease develops maximum during humid conditions specifically with dense plant stands (Hanna and Wells 1989). Pearl millet leaf blast was negatively correlated with dry matter yield, green-plot yield, and digestible dry matter (Wilson and Gates 1993). In rice, it was reported that blast affects the reduction in the overall growth, leaf area, 1000 kernel weight, dry matter accumulation, and grain yield (Bastiaans 1993). Resistance to blast in pearl millet was derived from the *P. glaucum ssp. Monodii* accession in which the rust gene (Rr1) was also identified (Hanna et al. 1987). Blast resistance in monodii accession was studied as three independent and dominant genes (Hanna and Wells 1989), though Tift 85DB, with resistance from monodii, was presented to have a lone resistance gene (Wilson et al. 1989) which is effective against all the tested *Pyricularia grisea* isolates. Several other sources of *Pyricularia* leaf spot resistance have been identified from Burkina Faso landraces. Each has been characterized as having dominant, single-gene resistance that is independent of the *monodii* resistance gene. Three pearl millet-segregating populations were screened for RAPDs (random decamer) primers and RFLPs using a core set of probes which detects single-copy markers on the map. In this study, one RAPD marker (OP-D11700, 5.6 cM) was linked to *Pyricularia* leaf spot resistance, and two other RFLP markers were linked to rust resistance genes (Morgan et al. 1998). In finger millet, Babu et al. (2014) developed 58 functional SSR markers for crucial blast resistance genes by using comparative genomic analysis with rice for population structure, genetic diversity, and association mapping approaches. Recent advancements in the pearl millet genome sequencing of reference genotype Tift 23D2B1-P1-P5 and resequencing of 994 pearl millet genotypes (Varshney et al. 2017) could be a great resource for breeders and genomics researchers, and will ultimately accelerate the efforts for identifying and mapping the resistant genes of important biotic and abiotic stresses. Two hybrid parent lines such as ICMB 06444 and ICMB 97222 were shown high level of blast resistance (Sharma et al. 2013.). These lines can be used for the development of high-yielding, blast-resistant pearl millet hybrids in India. Recently, hybrid HHB 146 has shown a high level of blast resistance by introgression of LG4 blast resistance QTL from 863B-P2 (Fig. 11.7).

Fig. 11.7 Blast-resistant LG4 QTL introgression hybrid HHB 146 improved (right) against the original blast susceptible hybrid HHB 146 (left)

11.4.4 Ergot and Smut

Ergot and smut are other fungal diseases in pearl millet. These two diseases are tissue specific which mainly infect the floral part of the plant (Thakur and Williams 1980). The causal agent of this disease (ergot disease) is *Claviceps fusiformis*, an ascomycete fungus, and symptoms of ergot include honeydew (cream to pink mucilaginous droplets) oozing from infected florets of panicle; these droplets contain asexual spores called conidia. This disease not only reduces the yield but also poisonous to humans, chicks, birds, and other animals (Kumar and Manga 2010). Ergot resistance is a recessive polygenic trait with notable cytoplasmic and nuclear interaction. The hybrid to be resistant for ergot, both parents, needs to carry the similar resistant alleles (Rai and Thakur 1995; Thakur and Rai 2003).

Smut is also an important floral disease of pearl millet in India, Western Africa, USA, and other countries growing pearl millet. It is caused by *Tolyposporium penicillariae* and reported to cause 5–30% yield losses in farmers' fields (Rachie and Majmudar 1980). So far there is no epidemics that have been reported, and the extent of yield losses caused by smut disease is quite variable (Thakur and King 1988). Panicle which is infected by the disease appears green, shining smut sori in place of grains on panicles; the sori mature within 2 weeks, turn into brown, and burst to release dark-brown to black spores which further infect healthy ear heads. Few studies reported that the resistance to smut is dominant and simply inherited (Phookan 1987). In another study, Chavan et al. (1988) reported that both dominant and additive gene effect for smut resistance, but the additive genetic effect was larger than dominance effect.

11.5 Future Prospects

In the current scenario of changing and un-predictive climatic conditions/events and global warming, pearl millet is going to be one of the crops of the future. Recent advances in the genetic and genomic resources of pearl millet have put it at par with other cereals like rice, maize, and wheat. ICRISAT led whole genome sequencing of the reference line and re-sequencing of nearly 1000 lines from all over the globe, and availability of more than 38 million SNPs on the world association mapping panel (PMiGAP) has thrown immense opportunities to mine and map novel alleles for both biotic and abiotic stress tolerance QTLs/genes. These newly developed ddRAD and WGRS-SNPs can be used in forward breeding in the development of hybrid parental lines and populations and in marker-assisted backcross (MABC) breeding programs. Some of the newly developed EST and genomic SSR markers have also shown promise in deciphering heterotic gene pools in pearl millet (Ramya et al. 2017). Knowledge on heterotic gene pools in combination with high-throughput genotyping and precision phenotyping platforms for important biotic (downy mildew, blast, rust, ergot, smut, *Striga*) and abiotic (drought, heat, salinity, low soil fertility) constraints may lead to an efficient pipeline for faster development of hybrid parental lines and improved populations suited to specific agroecologies of India, Africa, and other parts of the world. Pearl millet improvement programs need to constantly match itself with the evolving biotic and abiotic constraints. There is a need to keep pace with the shifting regional priorities as well. The genomics and breeding pipelines need to be constantly upgraded and aligned to deliver improved hybrid parental lines (A-/B- and R-lines) and populations tailored specifically to match the needs of specific agroecologies globally. An integrated approach combining modern genomic, molecular, statistics and data management tools and technologies is needed of the hour to expedite the breeding cycle to accelerate the rate of genetic gains by enhancing the yield and yield stability under stress.

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Chapter 12
Genomic-Assisted Enhancement in Stress Tolerance for Productivity Improvement in Sorghum

Kirandeep K. Romana, Girish Chander, Santosh Deshpande, and Rajeev Gupta

Abstract Sorghum [*Sorghum bicolor* (L.) Moench], the fifth most important cereal crop in the world after wheat, rice, maize, and barley, is a multipurpose crop widely grown for food, feed, fodder, forage, and fuel, vital to the food security of many of the world's poorest people living in fragile agroecological zones. Globally, sorghum is grown on ~42 million hectares area in ~100 countries of Africa, Asia, Oceania, and the Americas. Sorghum grain is used mostly as food (~55%), in the form of flat breads and porridges in Asia and Africa, and as feed (~33%) in the Americas. Stover of sorghum is an increasingly important source of dry season fodder for livestock, especially in South Asia. In India, area under sorghum cultivation has been drastically come down to less than one third in the last six decades but with a limited reduction in total production suggesting the high-yield potential of this crop. Sorghum productivity is far lower compared to its genetic potential owing to a limited exploitation of genetic and genomic resources developed in the recent past. Sorghum production is challenged by various abiotic and biotic stresses leading to a significant reduction in yield. Advances in modern genetics and genomics resources and tools could potentially help to further strengthen sorghum production by accelerating the rate of genetic gains and expediting the breeding cycle to develop cultivars with enhanced yield stability under stress. This chapter reviews the advances made in generating the genetic and genomics resources in sorghum and their interventions in improving the yield stability under abiotic and biotic stresses to improve the productivity of this climate-smart cereal.

Keywords Genomics · Markers · Molecular breeding · Nutrition · Sorghum · Stress tolerance

K. K. Romana · G. Chander · S. Deshpande (✉) · R. Gupta (✉)
International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India
e-mail: s.deshpande@cgiar.org; g.rajeev@cgiar.org

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12.1 Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is one of the most important robust and reliable cereal crops globally to feed around 500 million people who rely on it as a dietary staple (Dicko et al. 2006; ICRISAT 2018). It is the most common staple food in many semi-arid and tropical regions of the world because of its better adaptability and productivity under environmental stresses like droughts and high temperatures as compared with other cereals, namely, wheat, rice, and maize (Nedumaran et al. 2013; Reddy et al. 2012). Sorghum is often a multipurpose crop with dual benefits from stover as well as grain. Grain is used mainly as food or feed, whereas stover is a vital source of fodder for livestock. More than 35% of sorghum produced all over the world is used for human consumption and the rest as animal feed and production of alcohol and industrial products (Awika and Rooney 2004; Dicko et al. 2006). In sub-Saharan Africa (SSA) and South Asia (SA), sorghum grain is used as both human food and animal feed (including as poultry feed), whereas in the USA, Australia, Brazil, and other developed nations, it is used fundamentally as animal feed. With focus to address micronutrient malnutrition in about 2 billion people globally, promotion of crops, varieties, and agricultural practices that promote easy access of nutrient-dense food to rural masses is the most effective strategy (Wani and Chander 2016). In this context, sorghum along with pearl millet outshines as a choice crop having high contents of macro/micro-nutrient contents and meeting the nutritional requirements of people in SSA and SA. Improving the productivity and access through production in large areas, this crop could be an effective means to address undernourished children and women worldwide. Besides having important human health and nutritional qualities, sorghum is also a gluten-free cereal which makes it an alternate source of specialty foods for people with celiac disease who are allergic to gluten (Dicko et al. 2005; Rooney 2007).

Globally, sorghum is grown in about 42 million hectares area with a total production of 63 million tons from the year 2010 to 2016, across 98 countries in Africa, Asia, Oceania, and the Americas. Nigeria, India, the USA, Mexico, Sudan, China, and Argentina are the major sorghum-growing countries (Fig. 12.1). Other sorghum-producing countries include Burkina Faso, Chad, Ethiopia, Gambia, Ghana, Mali, Mauritania, Mozambique, Niger, Senegal, Somalia, Tanzania, and Yemen (FAOSTAT 2016). In countries like India, the productivity of sorghum in the year 2013 was reported to be quite low at about 850 kg per ha (FAO 2013) which suggest a huge potential to explore untapped genetic diversity in combination with modern genetics and genomics tools.

In India, the major sorghum-growing states are Karnataka, Maharashtra, Telangana, Andhra Pradesh, Madhya Pradesh, Tamil Nadu, Gujarat, and Rajasthan. In India, though, there is a decline in the sorghum cultivation area among all states, a significant increase in the productivity has been recorded. However, with increasing realization of global warming inducing climate change and increased health consciousness, focus on "climate-change ready" and nutritious crops like sorghum is regaining. As a consequence, in the USDA report (2016), an increase in the area

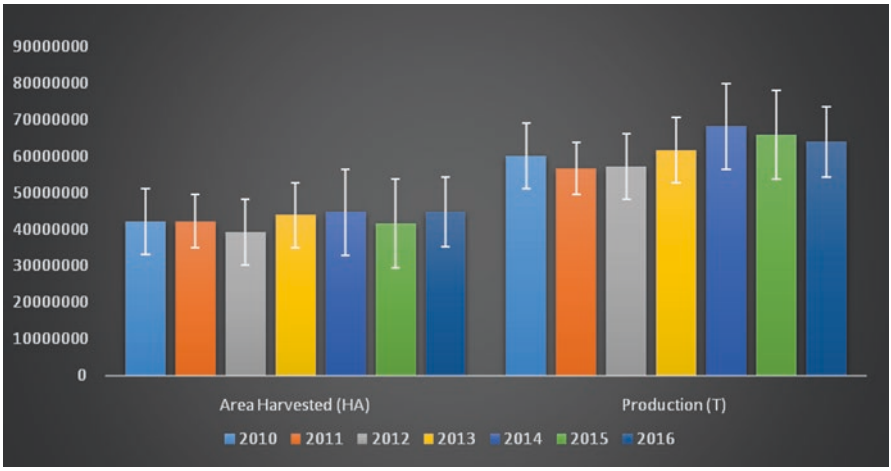

Fig. 12.1 Global sorghum productivity from the year 2010 to 2016. (Source: FAOSAT 2016)

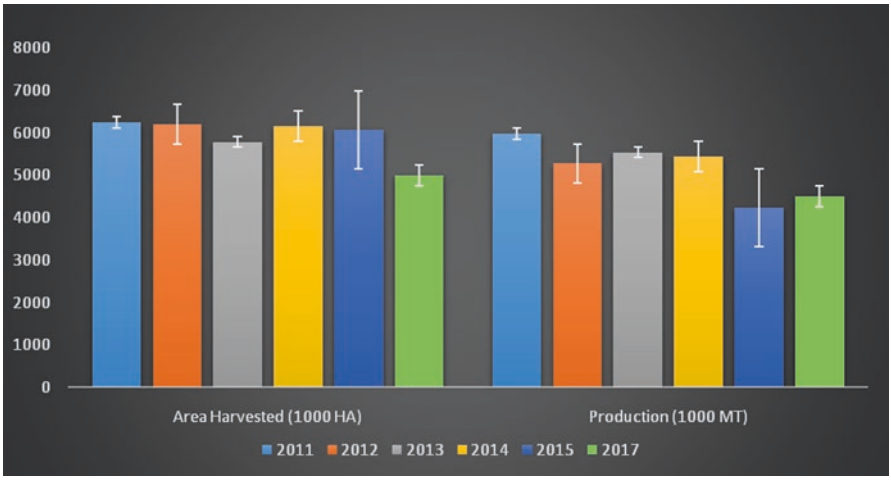

Fig. 12.2 Sorghum productivity in India from the year 2011 to 2017. (Source: USDA 2017)

of sorghum production by 19% and increase in grain harvest by 23% were observed globally (USDA 2016).

Sorghum production worldwide for the last 30 years revealed a declining trend in area under cultivation from 44.5 to 41.9 million hectares with corresponding decrease in production from 62.8 to 59.3 million metric tons (FAO Stat 2016). In India sorghum was grown in about 6245 (‘000 ha) in the year 2011 which has been reduced to 5000 (‘000 ha) during the year 2017 (Fig. 12.2). Similarly, sorghum production in India also showed decreasing trends from 5979 (1000 Mt) in the year 2011 to 4500 (1000 Mt) in the year 2017(USDA report 2017) (Fig. 12.2). In the present scenario of increasing effects of climate change on various crops and

regions, sorghum stands a great chance to adapt to several abiotic and biotic stresses and ensure future food and nutritional security globally as well in regions with perennial chronic malnourishment and undernourishment.

Genetic studies and conventional plant breeding research coupled with other crop improvement interventions were able to address many of the biotic and abiotic stresses over the last half of the century. The current climatic change scenarios have exposed the sorghum crop to variations in diurnal temperature regimes and variations in rainfall patterns that have not been experienced before. The sorghum breeding research is trying to cope with these new challenges; the advances in new biological technologies including especially genomics have the potential to make these adjustments at a faster pace, with improved efficiency and in most cases at relatively lower investments. Here we reviewed the recent updates from genomics research for addressing stress tolerance improvement.

12.2 Genetic and Genomic Resources in Sorghum

Large numbers of genetic, genomic, translational, and mutational resources had become available in the recent years for sorghum, which further raises the research opportunities for sequence variations in relation to the phenotypic traits of interest and their further productive utilization in the sorghum improvement programs. The recent applications of the molecular markers and genomic technologies have shown promising effects in efficient breeding. However, the success of sustained gains in any crop relies on the variation of crop gene pool. The ICRISAT genebank maintains 41,023 sorghum accessions assembled from 93 countries which include land races, breeding material, advanced cultivars, and wild relatives (Upadhyaya et al. 2018). The core (~10% of total collection) and mini-core (~1% of total collection) representative and reference sets have been developed at ICRISAT for future research endeavors. These sets were further used to identify new sources of variations for stress resistance, phenology, seed yield, quality traits, etc. Great opportunities for the genetic improvement of sorghum are made available via reference genome sequence (Paterson et al. 2009), and diverse sorghum lines were further compared with a reference genome that leads to substantial untouched diversity. Access to large number of markers including genomic and EST-SSRs, DArTs (Mace et al. 2009; Ramu et al. 2010), and alignment of major trait genes and quantitative trait loci (QTL) to integrated linkage and physical map (Mace and Jordan, 2011) had fast-tracked integration of molecular marker technologies to dissect complex traits such as stress tolerances. With rapid advancements in next-generation sequencing (NGS) technologies, identification of large number of markers, especially single nucleotide polymorphism (SNP), has become cheap as compared to the other marker systems, especially wet lab-based assays. These NGS assays (such as genotyping-by-sequencing, GBS) identified large number of SNPs across genome and provide opportunities to identify SNPs present closest to or inside the genes associated with target traits. These identified SNPs can be converted to customized SNP assays using several platforms such as CAPS, KASP™ platform, or

their modifications. Most of these platforms running SNP assays are inexpensive for fast-track and efficient utilization in breeding program either to transfer this trait or track the trait in target breeding populations. This will greatly improve the efficiency of introgression of component traits underlying different *stress tolerance* mechanisms by reducing breeding cycles (for recurrent parent recovery) and further recombining these for development of improved *stress*-tolerant cultivars. The recent examples of NGS application included genetic diversity and phylogenetic studies and trait mapping using genome-wide association mapping studies (GWAS). Morris et al. (2013) led to the discovery of approximately 265,000 single nucleotide polymorphisms (SNPs) in 971 worldwide accessions across Africa and Asia (which included the mini-core set, reference set and US sorghum association mapping panel) (Fig. 12.3). These findings were further reformed to diverse agroclimatic conditions. This phylogenetic study revealed that accessions cluster preferentially according to their geographic origin, followed by clustering into races or morphotypes. Utilizing the NGS technologies (Mace et al. 2013), resequenced 44 sorghum genotypes in which over 4.9 million SNPs were identified in *sorghum bicolor*. By utilizing gene-based population summary statistics of a prior selected candidate genes, 725 candidates were identified for domestication or improvement in sorghum (Mace et al. 2013). Recent initiatives of resequencing several hundred sorghum accessions such as TERRA-REF project (http://terraref.org/#genomic-and-genetic-data-and-computational-platform) targeting resequencing 1000 sorghum accessions for characterization of genetic variation and identification of genomic regions controlling biomass, plant architecture, and photosynthetic traits, and TERRA-MEPP project (https://terra-mepp.illinois.edu/team/ profile/michael-gore) will develop quantitative trait loci (QTL) models to predict daily plant biomass yield throughout the growing seasons.

Application of NGS tools like GbS for dissecting complex traits such as stress tolerance on DNA sequence level will capture most of the functional factors of genome related to trait expression. But other applications of NGS tools in RNA sequencing (commonly referred as RNA-seq) will help to capture the regulatory parts (Ozsolak and Milos 2011). For a complex trait such as stress tolerance/resistance, involving host-pest and/or environment interactions, many growth and development pathways are involved for its expression. Application of RNA-seq platforms can help understand the role of regulatory and transcription factors (including small RNA, microRNA) and their interaction with other pathways. There is big interest to utilize recent advances in RNA-seq technologies with the recombinants identified from fine-mapping exercise to move towards better understanding the stress tolerance in sorghum. Knowledge of QTL underlying stress tolerance traits can aid detection of resistance/tolerance genes (complex ➔ QTL) in sorghum.

Several genetic resources including, (i) germplasm panels such as mini-core set (Upadhyaya and Gowda 2009), sorghum reference set (Billot et al. 2013), and US sorghum association mapping panel (SAP); (ii) mapping populations such as random inbred line populations (over 10 RIL populations at ICRISAT-HQ, Patancheru; *Personal communication with Santosh Deshpande*) for several traits (Mace et al. 2013); (iii) nested association mapping (NAM) (Bouchet et al. 2017); (iv) backcross-based NAM (BCNAM) population, which can serve as genetic and breeding

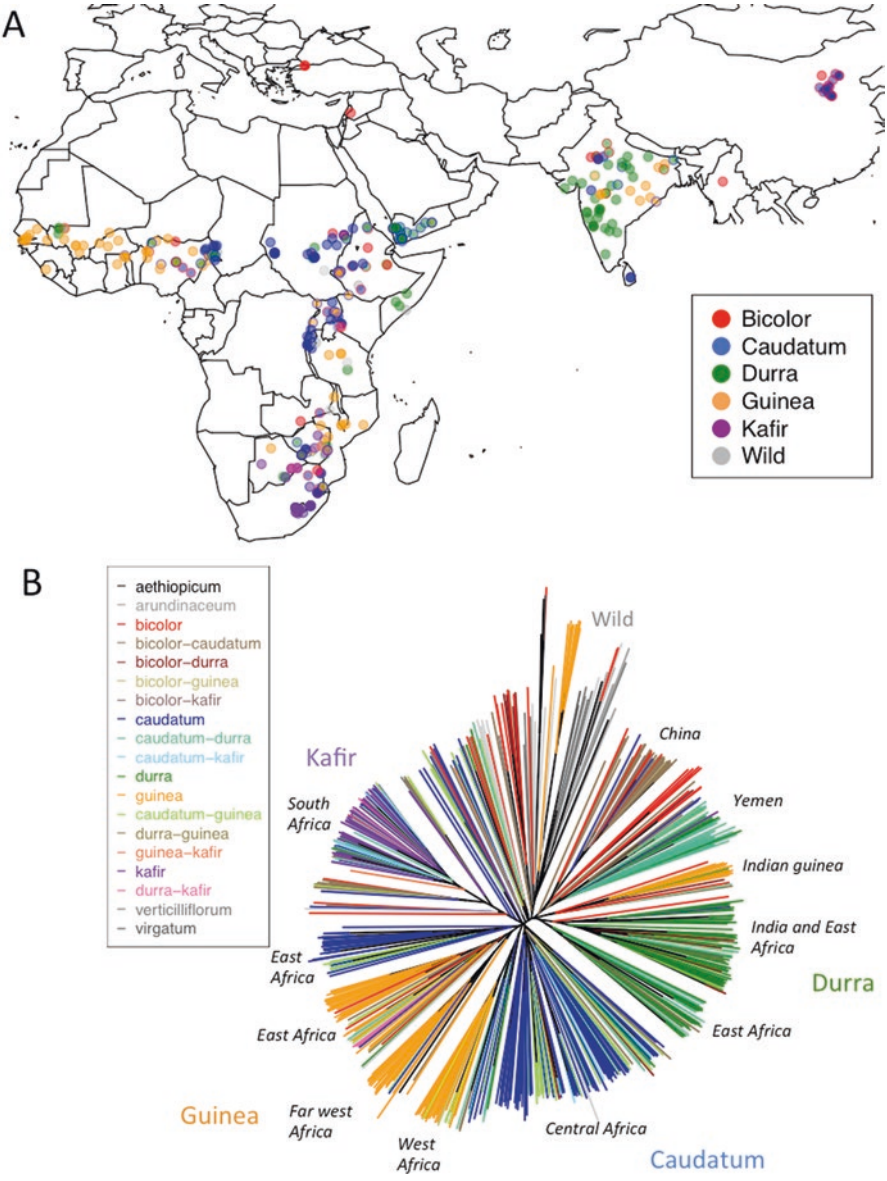

Fig. 12.3 Geographical and racial distribution of genetic diversity of sorghum accessions. (**a**) Geographic origin of 469 genetically diverse sorghum accessions studied by Morris et al. (2013); (**b**) neighbor-joining graph of the same 469 lines clustered into morphotypes within a region. (Source: Morris et al. (2013))

populations (Jordan et al. 2012); and (v) mutation populations such as TILLING population (Skelton 2014), are now available in sorghum. All these new tools play a major role in enhancing the breeding efficiencies by not only exploring genetic mechanisms but also providing or inducing new diversity in relatively elite backgrounds. These next-generation genetic resources are excellent tools for studying the connection between phenotype to genotypic variations. The BCNAM populations are already well established (Jordan et al. 2011) to benefit both trait mapping and introducing diversity that was not available previously in the traditional plant breeding populations. In sorghum improvement program at ICRISAT, BCNAM approach, in combination with other genomics tools, is currently being used extensively (Deshpande, unpublished) to address the priority traits related to yield, biotic and abiotic stress tolerance, etc. In terms of applications of these tools in molecular breeding, the NGS-based genotyping platforms such as whole genome resequencing (WGRS), genotyping-by-sequencing (GbS), etc., have the potential for molecular breeding (MB) and genomic selection (GS) which are emerging prediction tools in modern breeding programs. Considering the different levels of advancement of constituent technologies involved in GS, an appropriate resource investment strategy for every single breeding program needs to be developed for maximizing the rate of genetic gains. These resources provide the test bed for all genomics studies and their application for translational research for trait introgression and breeding applications.

12.3 Genomic Interventions for Abiotic Stress Tolerance

The conditions prevailed by abiotic stresses lead to extensive yield losses to agricultural production worldwide. Many researchers are instigating intensive research on various abiotic stresses such as drought, heat stress, and salinity (Bray et al. 2000; Cushman and Bohnert 2000). However, due to abiotic stresses, there is loss in the productivity of the crop to several degrees, and the losses were dependent on the time of onset, longevity, and stress intensity. Although sorghum is a harsh crop yet, drought is an important abiotic stress for sorghum. Other most important abiotic stresses in present era during cultivation are nutrient deficiency, aluminum stress, water logging, or temperature stress. The plants have to cope with all of them during cultivation along with high salinity and drought (Tari et al. 2012). The following section discusses some of major abiotic stresses in sorghum production.

12.3.1 Drought

Sorghum, one of the most drought-tolerant crops, is a dual-purpose crop. A detailed status on genomic interventions in abiotic stress tolerance in sorghum has been reviewed by Wang et al. (2014a, b). Water stress is the most important factor under

climate change affecting the crop productivity (Araus et al. 2002). In the past, many positive efforts were made for improving tolerance of sorghum to drought through genetic enhancement (Mutava et al. 2011). Two different types of stress responses were observed in sorghum at distinct time interval, i.e., pre-flowering and post-flowering drought response (Harris et al. 2007). In sorghum, the green leaf area retention during the grain-filling stage has been found to be linked with sorghum post-flowering drought tolerance (Rakshit et al. 2016). As reported by Deshpande et al. (2016), seven sources for stay-green trait in sorghum have been used for QTL identification, such as B35 (Tuinstra et al. 1997; Crasta et al. 1999; Subudhi et al. 2000; Xu 2000a, b; Sanchez et al. 2002; Harris et al. 2007), E36–1 (Haussmann et al. 2002), QL41 (Tao et al. 2000), SC56 (Kebede et al. 2001), 296B (Srinivas et al. 2009), SC283 (Sabadin et al. 2012), and SDS 1948–3 (Habyarimana et al. 2010). A consensus map for the sorghum chromosome SBI-01 to SBI-05 (Fig. 12.4a) and SBI-06 to SBI-10 (Fig. 12.4b) exhibits the QTL intervals for stay-green QTLs from Mace and Jordan (2011) which were further aligned to

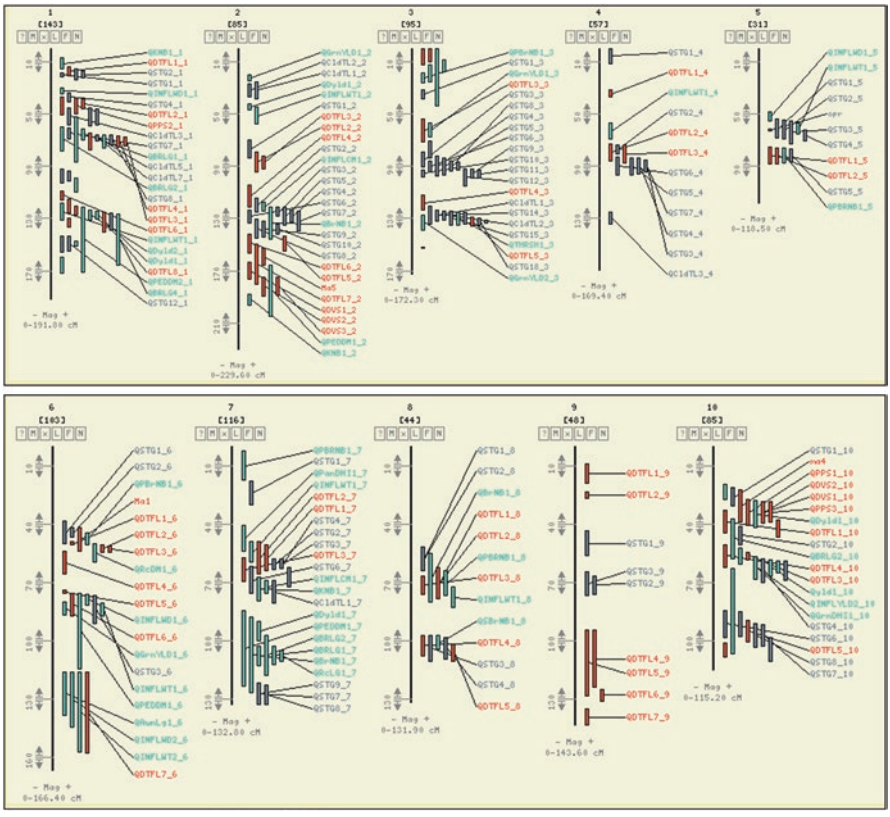

Fig. 12.4 (**a**) QTL tracks for sorghum chromosome SBI-01 to SBI-05. (**b**) QTL tracks for sorghum chromosome SBI-06 to SBI-10

physically aligned SSRs; the vertical bars in the map indicate the QTL interval tracks. The red color tracks in the map show up for the maturity genes/QTLs, and the aqua color is associated with panicle traits and gray color tracks for the stay green.

Stay-green QTL introgression research by Deshpande et al. (2016) was initiated at ICRISAT-HQ, Patancheru, India. This study led to the dissection of the genetic function of the stay-green QTLs, which suggested that *stg3A* and *stg3B* QTLs are responsible for transpiration efficiency and vapor pressure deficit response. In total, six QTLs were investigated, of which *stg3A* and *stg3B* QTLs were found to be more stable across genetic backgrounds and environments. The *stg3A* and *stg3B* QTLs are located next to each other on SBI-02, and the mesocarp gene, *Z*, which governs the grain seed coat color, is located in-between the two QTL intervals (Mace and Jordan (2010). For identification of the SNPs representing this targeted region in the sorghum genome, additional efforts are being made at ICRISAT (Deshpande et al. 2016).

For investigating the effects of stay-green introgression on stover fodder traits and grain-stover relationships, introgression of stay-green QTLs into sorghum S-35 and R-16 genetic backgrounds was executed at ICRISAT-HQ. Recently Blummel et al. (2015) have reported that one stay-green QTL (StgB) in S-35 increased the stover in vitro *organic matter digestibility* (IVOMD) along with grain and stover yield. Thus, a conclusion was drawn from the research that stay-green QTL can contribute to improve stover quality as well as grain and stover yield (Blummel et al. 2015).

For improving tolerance of post-flowering moisture stress in grain sorghum, a number of QTLs associated with stay green have been identified in sorghum for facilitation of its transfer for further adaptation. In the past few years, much research has been done for QTL discovery and consistency for stay-green traits, among these six major QTLs reported for stay-green traits, i.e., *stgC* (SBI-01), *stg3A* and *stg3B* (on SBI-02), *stg1* and *stg2* (on SBI-03), and *stg4* (on SBI-05) (Subudhi et al. 2000; Borrell et al. 2014). Detection, development, and influence of QTLs responsible for sorghum yield under drought tolerance were accomplished by many researchers (Tuinstra et al. 1998; Kebede et al. 2001). A very recent 2017 research, conducted by Jabereldar et al. (2017), on drought focuses on deficit irrigation scheduling practices on seed yield and water use efficiency of five sorghum genotypes. The results in this study highlighted that water stress at eighth leaf stage reduced the following traits, and reduction of length, panicle weight, number of grains per panicle, 100-grain weight, seed yield, and water use efficiency were also observed. There are many existing reports stating the presence of QTLs related to grain yield in sorghum for drought and cold tolerance or yield components (Sabadin et al. 2012; Phuong et al. 2013; Reddy et al. 2014; Kapanigowda et al. 2014). Also physiological parameters such as leaf greenness (chlorophyll content and chlorophyll fluorescence) play a pivotal role in its positive correlation to the grain yield under different conditions in sorghum (Xu 2000a; Harris et al. 2007).

12.3.2 Nitrogen Use Efficiency (NUE)

Nitrogen demand in agriculture is increasing across the world and is expected to reach up to approximately 250 million tons by the year 2050 (www.fao.org). It is well documented that almost 50% of the total nitrate applied is lost in the process of leaching, runoff and denitrification. This nitrate loss ultimately puts pressure on the cost of the crop production and lead to the pollution of water, soil and also contributes to greenhouse gas emissions. Therefore, development of genotypes or the crops with improved N absorption and utilization can lessen the problems of modern agriculture. With reference to nitrogen use efficiency (NUE), significant differences had been reported in sorghum genotypes (Maranville et al. 1980; Youngquist et al. 1992). Four N-tolerant sorghums, i.e., San Chi San, China17, KS78, and high-NUE bulk, and three sensitive genotypes such as CK60, BTx623, and low-NUE were used for bulk expression analysis studies under low N and normal N levels which revealed increased response of sensitive genotypes toward DEG transcripts associated with stress. However, tolerant genotypes showed increased root mass for efficient uptake of nutrients (Gelli et al. 2014). A population of 131 RILs derived from across between CK60 (inefficient N user) and China17 (efficient N user) were genotyped using GbS (Gelli et al. 2016), and multiple traits were found to be governed by the co-localized regions on chromosomal segments: SB-01, SB-05, SB-06, SB-07, and SB-09. The genomic regions of cloned QTLs and potential pleiotropic regions were equivalent for the genes associated with flowering time, *Ma3* on chromosome 1, and *Ma1* on chromosome 6, gene associated with plant height, *Dw2*, on chromosome 6. Further on the basis of the RNA sequencing data which represents the differential expression of transcripts related to nitrogen metabolism, glycolysis, seed storage proteins, plant hormone metabolism, and membrane transport, it was inferred that the differentially expressed transcripts could be probable objectives or promising targets for sorghum improvement under limited N fertilizer through marker-assisted selection.

To explore native genetic variation in nitrogen use efficiency, diverse germplasm panels of sorghum in field and glasshouse were studied under CINTRIN (Cambridge-India Network for Translational Research in Nitrogen) project. The researchers and scientists are evaluating a diverse set of almost 250 sorghum accessions (including parents of backcross-derived nested association mapping (BCNAM) populations; biparental mapping populations, etc., along with accessions from different countries such as Nigeria, China, the USA, Lesotho, Ethiopia, and Mali). The trial was laid in an alpha-lattice split-plot design with three replications per genotype per N dose (with three doses of 0% N, 50% N, and 100% N of recommended nitrogen (90 kg per hectare) at ICRISAT, Patancheru. The trial was laid with 15 cm × 75 cm spacing between plants in a row and between rows, respectively, involving 60 diverse sorghum accessions adopted for short-day conditions (Fig. 12.5). The preliminary results showed the native variation to N response among accessions with possibility of identification of accessions with variable N response.

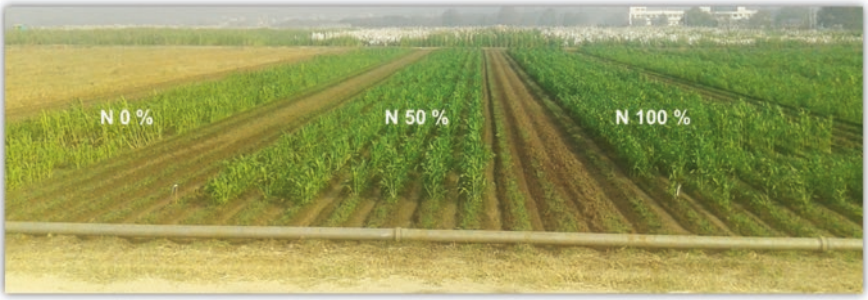

Fig. 12.5 Differential N response of sorghum accessions in field evaluation at ICRISAT-HQ, Patancheru, India, during post-rainy season of 2016–2017

12.3.3 Aluminum (Al) Tolerance

One of the constraints for the sorghum crop productivity in the tropics and subtropics, especially in West Africa, is the acidic soils (Wang et al. 2014a, b). The plants or the crops growing under the Al toxicity exhibit stunted growth and are often susceptible to drought (Marschner 1991; Kochian et al. 2004; Wang et al. 2014a, b). A single gene (AltSB/SbMATE) controls the Al tolerance in sorghum (Magalhaes et al. 2004) that has been mapped to sorghum chromosome 3. However, Magalhaes et al. (2007) have identified a gene coding for aluminum-activated citrate transporter via positional cloning, and also the markers from the similar region have been used by sorghum breeders to introgress favorable SbMATE alleles in susceptible sorghum genotypes (Anami et al. 2015).

12.3.4 Heat Stress

Air temperatures have been warming in most of the major cereal cropping regions around the world in the past decades (Lobell and Gourdji 2012). The risk of complete crop failure due to sterility during the critical reproductive period of heat stress was reported by (Teixeira et al. 2013). The average yields were ≤ 1.0 t ha^{-1} due to negative impacts of these stresses. Moreover, climate change factors are projected to impact the sorghum yields considerably especially for short duration of high temperature (above optimum) (Prasad et al. 2008). For growing sorghum, the mean optimum temperature range for grain sorghum is 21–35 °C for seed germination, 26–34 °C for vegetative growth and development, and 25–28 °C for reproductive growth (Maiti 1996). Sorghum-producing regions often experience daytime/nighttime temperatures of >32 °C/22 °C (Prasad et al. 2006). The effect of high-temperature stress causes significant decrease in sorghum grain yields as reported by Prasad et al. (2006). For grain sorghum, the dry matter and seed yields were maximum at 27 °C/22 °C (daytime/nighttime temperature), and temperatures above 33/28 °C

during early stages of panicle development induce floret and embryo abortion (Downes 1972). Several reports have shown decrease in seed-filling duration, resulting in smaller seed size and lower seed yields due to high temperatures (Chowdhury and Wardlaw 1978; Kiniry and Musser 1988). In a study conducted by Prasad et al. (2006), season-long (from emergence to maturity) effects of a range of high temperatures (HT) >35/25 °C on physiology, growth, and yield of grain sorghum hybrid DK-28E were quantified, and also due to HT (≥36/26 °C), there was significant decrease in the seed set, seed number, seed size, seed-filling duration, and seed yields when compared with optimum temperature (OT) (32/22 °C). At the seedling stage in terms of poor emergence, plant death and reduced plant stands, sorghum was observed to be sensitive (Kumar et al. 2011). For heat tolerance in sorghum, genetic variability was observed (Sullivan and Blum 1970; Blum and Ebercon 1976; Sullivan et al. 1977; Sullivan and Ross 1979; Jordan and Sullivan 1981).

Reports have shown the effects of high-temperature stress which are more prominent on reproductive development than on vegetative growth, and the sudden decline in yield with temperature is mainly associated with pollen infertility in many crop species (Young et al. 2004; Zinn et al. 2010). A set of 18 diverse sorghum genotypes were used to explore genotypic variation in high-temperature tolerance where plants were grown in a controlled environment facility under four conditions ranging in maximum temperatures from 32 °C to 38 °C but had similar minimum temperature at 21 °C and relative humidity throughout the day ranged between 52% and 94%. Results have shown that high temperature significantly accelerated development and reduced plant height but had no consistent effect was observed on the leaf area per plant. In vitro studies conducted on pollen germination percentage and seed set percentage were significant for all genotypes. Pollen germination percentage and seed set percentage were observed to be highly correlated, for subset of six genotypes, and seed set percentage in controlled environments correlated well with that in field experiments that experienced similar temperatures around anthesis. These results indicated that seed set percentage could be a useful phenotypic screen for high-temperature tolerance (Nguyen 2014). These observations indicated that seed set percentage could be a useful phenotypic screen for high-temperature tolerance in sorghum. Screening for seed set percentage in plants subjected to high temperatures around anthesis could provide a phenotypic screen for high-temperature tolerance that could be an implementation in crop improvement programs.

12.4 Genomic Interventions for Biotic Stress Tolerances in Sorghum

Various biotic stresses affect sorghum leading to a severe reduction in productivity and production in various production systems (Kumar et al. 2015). For overcoming these biotic stresses, combination of genetic and management practices could be an effective means to alleviate the problem up to some extent, but the development of

insect-resistant high-yielding varieties for farmer cultivation had not been commenced effectively. One reason for this lacuna could be lack of knowledge on inheritance of agronomic and morphological characteristics allied with insect resistance and yield of the crop (Sharma et al. 2005; Riyazaddin et al. 2015). For reduction of plant biotic stress from insect attacks, late planting could be an option (Li et al. 2007). Various diseases such as anthracnose, charcoal rot, etc., have been reported in several years affecting the sorghum productivity.

Globally, sorghum is reported to be damaged by over 150 insect species triggering the predictable annual loss of more than US$ 1000 million (Sharma et al. 2003). For sorghum productivity and production, the major biotic constraints are insects and pests such as shoot fly (*Atherigona soccata*) that is often considered to be the major yield limiting factor causing damage to the late sown sorghum crop. In addition, stem borer and aphids are insect-pests causing severe crop damage at later growth stages. The following section briefly discusses the major biotic stresses for sorghum production.

12.4.1 Shoot Fly

Grain and fodder yield losses in sorghum are mostly due to shoot fly infestation which results in decreased plant stand of the crop. The global yield loss due to shoot fly has been reported to be approximately over than US$ 274 million (Sharma 2006). The estimated losses due to this pest have been reported to reach as high as 86% for grain and 46% for the fodder yield (Syed et al. 2017). The yield loss in sorghum due to shoot fly occurs after the emergence of sorghum seedlings between the first and fourth week. The infestation of shoot fly occurs by oviposition on the leaf surface of the third and sixth basal leaves of the seedlings. The major loss was observed in the plant stand and the grain yield due to shoot fly increase on account of delayed planting exclusively in high-yielding cultivars of sorghum (Rai et al. 1978).

By the use of resistant varieties, seed treatment with systemic insecticides, planting in time, and need-based foliar spray applications during the seedling stages, the yield losses due to shoot fly can be evaded to a major extent (Sharma 1985). Also one of the major effective means is to have host plant resistance for the shoot fly populations keeping the poor farmers benefitted from the use of costly insecticides for shoot fly damage in the semi-arid zones (Sharma 1985; Sharma 1993; Riyazaddin et al. 2015).

Improvement in genetic makeup of sorghum crop considering the economic importance of this pest is a major objective of many sorghum breeding programmers. For a better understanding of resistance inheritance, QTL identification and the linked markers are important for successful introgression of the identified QTLs through marker-assisted breeding. Past reports and studies on sorghum shoot fly resistance recommend quantitative nature of the trait (Sajjanar 2002; Folkertsma et al. 2003). A total of 29 QTLs for 5 component traits of shoot fly resistance were

identified by Satish et al. (2009) in a 168 RIL mapping population derived from the cross 296B × IS18551 with a varying degree of phenotypic variation. Screening of 32 sorghum genotypes for shoot fly resistance has been accomplished, of which 16 genotypes were found to be resistant to shoot fly dead hearts (Prasad et al. 2015). Introgression of four validated QTLs imparting resistance for shoot fly at ICRISAT, governing major component traits such as dead hearts incidence, leaf trichome density, reduced oviposition, and leaf glossiness, was done into two elite genetic backgrounds of BTx 623 and 296 B through MABC (Ramu et al. 2010). The research aimed at discovering the genetic basis of resistance into QTL, using replicated phenotypic data sets obtained from 4 test environments, and a 162 microsatellite marker-based linkage map constructed using 168 RILs of the cross 296B (susceptible) × IS18551 (resistant) (Satish et al. 2009). Similar research was done by Aruna et al. (2011) for shoot fly resistance and associated traits that detected 25 QTLs (five each for leaf glossiness and seedling vigor, ten for dead hearts, two for adaxial trichome density, and three for abaxial trichome density) in individual and across environments. The QTLs identified in these researches will enable marker-assisted breeding for shoot fly resistance in sorghum in future breeding programs, and efforts have already been in progress toward this direction at ICRISAT.

12.4.2 Stem Borer

In different sorghum-growing regions all over the world, several species of stem borer attack were observed (Nwanze 1997) among which spotted stem borer (*Chilo partellus)* is predominant in Asia and eastern and southern Africa. Usually three types of stem borers that attack sorghum include the spotted stem borer, African maize stalk borer, and the African pink stem borer. Several strategies and methods are available for the management of stem borer as mentioned earlier besides chemical insecticides, such as biological control like introducing parasitoids (*Cotesia flavipes*) (Khan et al. 2003; Tende et al. 2005). However, there is no such treatment which could completely eradicate the stem borer damage, as biological methods are laborious and time-consuming and are not effective in the long run when already the damage had happened (Mailafiya et al. 2009). Chemical insecticide control, if applied before the damage, had inflicted on the crop as the most effective measure for stem borer control. But usage of chemical insecticides is too expensive and out of the reach of poor farmers. A viable option for the farmers would be the host plant resistance for the insect pest management in sorghum and also other cereals that will be cheaper, environmental friendly, and mostly compatible to other pest control strategies (Tadele et al. 2011). Indian national sorghum improvement program (whole world population screening) and International Crops Research Institute for the Semi-arid Tropics (ICRISAT) had screened over 30,000 world germplasm accessions for spotted stem borer resistance (Singh et al. 1968; Pradhan 1971; Jotwani 1978; Taneja and Leuschner 1984; Sharma et al. 1992, 2003). The studies done by Sharma et al. (2003) on the effect of spotted stem borer damage on fodder quality in sorghum resulted in the assessment of the losses associated with stem

borer in fodder yield and quality in sorghum. The study also revealed a very significant outcome of identifying a dual-purpose (fodder and grain) cultivar by the farmers for the stem borer-resistant sorghum genotypes (Sharma et al. 2003).

Stem borer resistance is a quantitative trait with little heritability (Singh et al. 2011). Inheritance additive genes were reported to be important for stem borer resistance (Karaya et al. 2009). When selections for *B. fusca* and *C. partellus* resistance were implemented, multiple traits such as exit holes, leaf feeding, stem tunneling, and dead heart were considered (Tadele et al. 2011). The factors in the cytoplasm of the nuclear genes and the maintainer lines influence the stem borer resistance (Sharma et al. 2007). In both the restorer and the male sterile line, high level of resistance is the prerequisite for production of stem borer-resistant hybrids (Dhillon et al. 2006). Inheritance of the markers and quantitative traits recognized can be used to select complex traits by enhancing the quantitative trait loci mapping (Bernardo 2008). For the damage due to stem borer in sorghum, genetic linkage maps are essential for resistance/tolerance (Sally et al. 2007). In cereals, many distinct QTLs for stem borer and agronomic traits had been mapped. For the resistance to European corn borer-stem tunneling, QTLs were mapped in RILs of "B73" × "DE811" (Krakowsky et al. 2004).

12.4.3 Anthracnose

One of the destructive fungal diseases in sorghum that causes high-yield losses is anthracnose, caused by *Colletotrichum sublineolum*. Anthracnose was first reported in sorghum from Togo in 1902. This disease presently reported worldwide (Bergquist 1973; Frederiksen 1984; Heald and Wolf 1912; Hsi 1956; Miller 1956; Pastor-Corrales and Frederiksen 1980; Porter 1926; Sundaram et al. 1972; Tarr 1962). The strategies for increasing production against fungal pathogens, genetic engineering, and classical breeding for the traits conferring tolerance and resistance could be a precise approach for decreasing the losses caused by them. All the areal parts of sorghum including tissues can be infested by anthracnose (Erpelding 2010). On all of the above ground tissues of the sorghum plant, anthracnose infection can be detected that includes seed, panicle, stalk, and leaf (Hess et al. 2002; Thakur and Mathur 2000). For anthracnose control, the most cost-effective measure is genetic resistance (Rezende et al. 2004).

In sorghum, many diverse sources of anthracnose resistances have been identified and were already mapped by several research groups from biparental crosses on chromosome 6 using an F5 mapping population (Klein et al. 2001). Another research by Mohan et al. (2010), QTL identification with a biparental mapping population of 168 F7 lines, was reported. Also Singh et al. (2006) on SBI-08 has identified markers for a recessive anthracnose resistance allele from 49 F8 inbred lines from mapping population. One more population association study for anthracnose resistance was performed by Upadhyaya et al. (2013) on sorghum mini-core collection (Upadhyaya and Gowda 2009). A RIL population was produced of 117

inbred lines by a study done by Burrell et al. (2015), and a total of 619 SNP and three microsatellite markers were generated for creating a genetic map for QTL analysis. This phenotyping study for anthracnose symptoms confirmed similar results for identified QTLs on chromosome 5, with the research of Cuevas et al. (2014) and Perumal et al. (2009). For detection of the resistance genes, molecular markers and QTL analysis play important role in carrying out marker-assisted selection for few fungal diseases. A recent study conducted by Patil et al. (2017) identified QTLs with different variable levels of resistance across all the tested environments. For marker-assisted introgression of confirmed anthracnose resistance genotypes into elite sorghum inbreds, single nucleotide polymorphisms linked to these loci will be very informative and a useful tool for future research (Patil et al. 2017).

12.4.4 Charcoal Rot/Stalk Rot

Charcoal rot also known as stalk rot is another major disease that occurs in sorghum-growing areas across the world. The charcoal rot is caused by *Macrophomina phaseolina* that affects the grain sorghum plant, as a result the sorghum panicle fails to fill the grain properly. In severe charcoal rot, the plants even may lodge in the later part of the season. Also fungal structures can be observed in the affected tissues that appear dusted with black pepper. The charcoal rot control management can be done by avoiding moisture stress, by balancing nitrogen and potassium fertility levels, and by growing drought-tolerant cultivars as well lodging resistant varieties, which could be some of the best means for its control. Modern biotechnologies have proved to be boon for such severe diseases as it provides DNA-based markers that could be genetically associated with economically important traits.

A very recent research done on charcoal rot by Reddy et al. (2008) had identified QTLs for the charcoal resistance in sorghum. This study had grown a F9 population consisting of recombinant inbred lines (RILs), derived from IS 22380 (susceptible) and E36–1 as resistant check in two locations of Dharwad and Bijapur in Karnataka, India. At both locations nine QTLs were identified for the component traits of charcoal rot disease (Dharwad, five QTLs, and Bijapur, four QTLs). The identified QTLs can further assist in marker-assisted selection (MAS) for charcoal rot resistance in sorghum. Another research done by Borphukan (2017) had revealed three QTLs for the charcoal or the stalk rot disease in sorghum. The study was carried out on the mini-core set of 242 sorghum accessions at ICRISAT and was evaluated with 2 charcoal rot check cultivars SPV 86 (susceptible) and E36–1 (resistant) under charcoal rot sick plot condition at MARS, Dharwad harboring charcoal rot resistance QTLs (qCr1, qCr2, qCr3). All the identified QTLs were found to be present in six sorghum accessions, i.e., IS4515, IS13549, IS29582, IS25301, IS12735, and IS23514. Another interesting observation was made that three resistant accessions (IS19389, IS17941, IS29233) and two moderate resistant accessions (IS1212 and IS32439) were found to carry the three qCr combinations.

12.4.5 Grain Mold

Another major constraint to sorghum productivity is the grain mold disease. This disease is also one of the most widespread diseases worldwide. Its effect is mostly observed in short duration hybrid cultivars and varieties, mostly grown in rainy season under humid conditions. The yield production loss due to this disease ranged from 30% to 100% depending on the cultivars, weather conditions, etc. (Singh and Bandyopadhyay 2000). However, exact estimate of the losses is difficult since it involves assessment from production to marketing and final utilization of the grain. The first visible symptoms of grain mold disease are pigmentation of the lemma, palea, glumes, and lodicules. The symptoms could be highly variable depending on the fungus involved at grain maturity stage. QTLs were reported with 130 markers including 44 SSRs and 85 AFLPs. Klein et al. (2001) reported that five QTLs for grain mold disease with 10 and 23% of the phenotypic variance in sorghum. In another glasshouse and field experiments, few QTLs were identified for grain mold disease for 242 sorghum mini-core accessions with 14,739 SNP markers (Upadhyaya et al. 2013).

12.5 Future Prospects

In the present era, data management and new genetics, genomics, statistics, and molecular resources, tools, and technologies have a great potential to enhance the efficiency of sorghum breeding program. The wide collection of germplasm, genetic, genomic, and breeding information/resources such as recent advancement in NGS, high-throughput phenotyping, next-gen breeding populations will enhance the rate of genetic gain in sorghum. Sorghum research community has access to large genetic (germplasm with unique traits) and genomic (SSRs, SNPs, high-density genetic maps, genome sequence) resources, and many QTLs/candidate genes associated with agronomic and stress tolerance traits are known in sorghum. The exploitation of a vast amount of untapped natural (primary, secondary, tertiary gene pools) and induced (TILLING, BCNAM, mutant populations, transgenics, etc.) genetic diversity could help sorghum improvement programs by mining alleles and controlling various biotic and abiotic stress tolerance. Rapid development of NGS technologies will further enhance identification of candidate genes and SNPs. The powerful combination of high-throughput genotyping and phenotyping could lead to molecular marker development and deployment for various biotic and abiotic stress tolerance traits to expedite the breeding cycle resulting in enhanced rate of genetic gains. The present era of research demands further advancement in sorghum via genetic engineering and gene transfer technologies for integration of desirable traits (without variation in cultivated gene pool) into the sorghum genome. Already many such reports are present in case of crops such as rice, wheat, and maize which has led to the development of several improved cultivars and advanced

lines that show greater yields, possess good grain quality, and minimize damage caused by pests and diseases. Improvement of sorghum with enhanced nutrition value combined with biotic and abiotic stress tolerance will at the end enhance economic benefits to the farmer and lead to augmentation of the quality and production in the coming years. The integration of these new tools along with new generation breeding populations will further help accumulating the required information to develop and deploy genomic selection for sorghum improvement. For all these initiatives, a greater investment from public/private organizations with well-defined partnership with farming community is the only way ahead.

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Chapter 13
Chickpea Genomics

C. Bharadwaj, Supriya Sachdeva, Rajesh Kumar Singh, B. S. Patil, Manish Roorkiwal, Sushil Chaturvedi, and Rajeev Varshney

Abstract As precise phenotyping is essential and the cost of generating phenotyping data at every generation is very expensive, recent advances in genomics technologies and the availability of a wide range of genotyping platforms have made the cost of genotyping much less expensive compared with phenotyping. The recent developments in sequencing technologies have manifold increased the repertoire of various types of markers that are available in chickpea including SSRs, SNPs, DArTs, hundreds of thousands transcript reads and BAC-end sequences saturated genetic maps, QTL maps as well as physical maps, and the sequencing of both kabuli and desi type has greatly helped in using marker-assisted technologies to be applied in plant breeding. Germplasm resequencing for identification of genome-wide SNPs and their subsequent utilization in genomic selection has the potential to break the yield barrier being experienced in chickpea and many other crops. Genomic-assisted breeding for marker-assisted backcrossing (MABC) for introgressing QTL region, marker-assisted recurrent selection, gene pyramiding, marker-assisted selection (MAS), and genomic selection can now be taken up in chickpea. The conventional plant breeding should take these tools to make greater genetic gains, increase selection potential, and have faster breeding cycles so that the genetic improvement gains are increased in chickpea.

Keywords Chickpea · Genomic-assisted breeding (GAB) · Marker-assisted backcrossing (MABC) · QTL · Marker-assisted recurrent selection (MARS) · Gene pyramiding · Marker-assisted selection (MAS) · Genomic selection

C. Bharadwaj (✉) · S. Sachdeva · R. K. Singh · B. S. Patil
Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India

M. Roorkiwal · R. Varshney
Centre of Excellence in Genomics, ICRISAT, Patancheru, India
e-mail: M.Roorkiwal@cgiar.org; R.K.Varshney@cgiar.org

S. Chaturvedi
ICAR-Indian Institute of Pulses Research, Kanpur, Uttar Pradesh, India

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13.1 Introduction

One of the most pressing problems of the world today is adequate nutrition for exploding human population as 870 million people go hungry worldwide (http://www.fao.org). The growing world population together with the lack of expansion or even reduction of available arable lands needed to maintain agricultural sustainability implies that the relative importance of plant breeding to raise crop yield potential and adaptiveness is now greater than in the past (Araus et al. 2002). Plant adaptation is a key factor that will determine the future severity of the effects of climatic change on food grain production. Relatively inexpensive changes, such as shifting planting dates or switching to an existing crop variety, may moderate the negative impact of climatic change. However, improvements in crop productivity to meet the requirement of growing demand mentioned above will not be easy without further technological breakthroughs that allow yield ceilings to be shifted through the development of new crop varieties (Rosenzweig and Parry 1994).

In India, from time immemorial, many legumes and pulses have been consumed as part of a primarily cereal-based diet. In the last 50 years, per capita availability of pulses has been steadily coming down from 70 grams/capita/day to 34 grams/capita/day (http://www.faostat3.fao.org). This is solely on account of stagnant crop yields. Annual pulse production has barely crawled in the last 23 years from 13.3 million tonnes in 1985–1986 to 17.8 million tonnes at present. On the other hand, for the majority vegetarian population of India, the sole protein supplement is getting out of reach with a nutritional calamity looming large. The major challenges impeding the pulse production and productivity are limited genetic diversity in the primary gene pool, genotype and environment interaction, multiple biotic and abiotic stresses, and limited screening methods for the precise phenotyping of the target traits (Kumar et al. 2014).

Chickpea, an oldest and widely cultivated pulse crop of the world, commonly known as Bengal gram or Garbanzo (*Cicer arietinum* L.). It is a highly self-pollinated crop, with $2n = 2x = 16$ (Arumuganathan and Earle 1991) and genome size of 738 MB with an estimated 28,269 genes (Varshney et al. 2013b). It belongs to genus *Cicer*, tribe Cicereae, family Fabaceae, and subfamily Papilionaceae. It originated in southeastern Turkey (Ladizinsky and Adler 1976). The name *Cicer* is of Latin origin, derived from the Greek word "*kikus*" meaning force or strength. Nutritionally, chickpeas contain 23% protein, 64% total carbohydrates (with 47% starch and 6% soluble sugar), 5% fat, 6% crude fiber, and 3% ash. It is also a rich source of vitamins, minerals like phosphorus, calcium, magnesium, iron, zinc and dietary fiber or Non-Starch Polysaccharides (NSP). Chickpea is grown widely for diverse uses throughout the Indian subcontinent, Mediterranean basin, the Middle East, and Africa and is becoming an important legume crop in new regions like Australia and North America, because of its nutritional value, diversified uses, and ability to grow better with low inputs under harsh edaphic and arid environments than many other crops. It is an important component of the cropping system of subsistence farmers in the Indian subcontinent, West Asia and

North Africa. Some of these attributes together with its ability to derive more than 70% of its nitrogen from symbiotic nitrogen fixation (Saxena et al. 1988) make chickpea a promising crop for sustainable agriculture.

Chickpea is a major *rabi* pulse with significant contribution toward pulse economy of the world. It is cultivated on 13.2 million hectares of land with 11.62 million tonnes of production across the world. India is the largest producer of chickpea with an area of 7.58 million hectares which produces 8.32 million tonnes of chickpea (FAOSTAT 2012; Agriculture statistics at a glance 2013). India accounts nearly 70% at a global level and 75% at Asian level in terms of world's chickpea area and production. In spite of being major producer, Indian productivity (912 kg ha^{-1}) is very low as compared to other major chickpea-growing countries like Myanmar (1407 kg/ha), Ethiopia (1549 kg/ha), USA (1825 kg/ha), Canada (1825 kg/ha), and Mexico (1500 kg/ha). Productivity enhancement in chickpea can help to release this negative trade balance as it contributes more than 45% to Indian pulse economy. Many approaches have been advocated for much needed chickpea productivity enhancement which can create additional genetic variation especially for traits of economic importance and enable effective utilization of available germplasm in chickpea improvement programs for enhanced and sustained chickpea production across the continents. Various biotic and abiotic stresses hamper chickpea production. Among the biotic stresses, *Ascochyta* blight, *Fusarium* wilt, *Helicoverpa* pod borer, and *Botrytis* gray mold are very important yield reducers. Drought, heat, cold, and salinity stress are the major abiotic factors that significantly affect chickpea yields.

As almost all the traits with agronomic values are genetically complex, which are affected by many genes, environments, and their interactions (Cramer et al. 2011; Grishkevich and Yanai 2013), identification of involved genetic factors such as quantitative trait loci (QTL) has been playing a vital role in manipulating the traits of interest and understanding of genetic architecture (Holland 2007; Xu 2010). However, conventional breeding requires assaying all the individuals for the target traits collected from a sample population making it expensive and time-consuming and needs to be supplemented with genomic-assisted breeding (GAB) (Varshney et al. 2005, 2007). Due to lack of chickpea genomic information until recently, it was considered an orphan legume for implementing GAB. On the other hand, recent advancements in the comparative genomics and genomic approaches have generated the genome sequence and genomic resources transforming chickpea to a resource-rich crop similar to other major food crops (Thudi et al. 2014).

13.2 Genomic-Assisted Breeding for Abiotic Stress Tolerance in Chickpea

13.2.1 MAS for Drought Tolerance

Being stable, unaffected by environment, and easily assessable with no disparity of growth and developmental stages, molecular markers are now considered ideal for diversity studies, QTL identification, fingerprinting, gene tagging, constructing

linkage maps, positional cloning, evolutionary studies, and marker-assisted selection (Bharadwaj et al. 2010; Shubha et al. 2011; Pooja et al. 2014; Chaudhary et al. 2014; Maqbool et al. 2016). The very first chickpea genetic map based on SSR markers was developed by Winter et al. (1999), and they reported 174 paired primers. NIPGR (The National Institute for Plant Genome Research, India) developed 280 SSR markers with the help of microsatellite enrichment (Sethy et al. 2006). SSR markers developed for chickpea have been used for genetic map construction (Radhika et al. 2007; Bhardwaj et al. 2002; Shefali et al. 2015), assessment of interspecific genetic diversity (Udupa et al. 1999; Bharadwaj et al. 2010; Yadav et al. 2011), QTL mapping for agronomic parameters (Udupa and Baum 2003; Subodh et al. 2015), and assessment of drought tolerance (Maqbool et al. 2016).

Chickpea accession ICC 4958 was used for development of novel 311 SSR primers (Nayak et al. 2010). Expressed sequence tags (ESTs) have also been mined for SSR primers (Varshney et al. 2009). Tentative unique sequences (TUSs) were used for designing 3172 SSR paired primers out of which 728 were nonredundant SSR paired primers, after identification of 26,252 SSR sequences (Hiremath et al. 2011). BAC libraries were used by Lichtenzveig et al. (2005) for development of 233 SSR markers. Thudi et al. (2011) designed 1344 SSR primers after identification of 6845 SSRs by mining of 46,270 BAC-end sequences. Heuttel et al. (1999) developed 28 SSR primers, Winter et al. (1999) developed 174 SSR primers, Sethy et al. (2003) developed 10, Sethy et al. (2006) developed 85, Qadir et al. (2007) developed 63, and Nayak et al. (2010) developed 311 SSR primers for chickpea. The recent genome sequencing project of chickpea enabled the discovery of 81,845 SSRs, of which 48,298 SSRs were found to be suitable for development of SSR primers for PCR amplification (Varshney et al. 2013b). These SSR markers are exclusively being used for improvement of drought tolerance in chickpea. "*QTL hotspot*" has been successfully introgressed into the genetic background of the elite varieties JG11, KAK2, and Chefe. Three SSR markers (TAA170, ICCM0249, and STMS11) were used for foreground selection, and 10 amplified fragment length polymorphism (AFLP) primer combinations were used for background selection after each generation of backcrossing while introgressing "*QTL hotspot*" into JG 11 genetic background. A total of 29 introgression lines were developed with ~93% recurrent parent genome recovery after three backcross cycles followed by two generations of selfing (Varshney et al. 2013c). The introgression lines developed from JG11 x ICC 4958 were found to possess higher root length density, root dry weight, and rooting depth compared to both the donor and recipient parents; these are the most important target traits for enhancing drought tolerance in chickpea (Varshney et al. 2013a, b). Furthermore, preliminary analysis of phenotypic evaluation of these lines in India (Patancheru, Dharwad, Nandyal, Durgapura, and Gulbarga), Kenya, and Ethiopia indicated that several lines with >10% increase in yield under rain-fed conditions and ~20% increase in yield under irrigated conditions were available. Based on the preliminary results, other national partners like IIPR, IARI in India, and Egerton University (Kenya) and the Ethiopian Institute of Agricultural Research (Ethiopia) in sub-Saharan Africa initiated introgressing this region into genetic backgrounds

of elite cultivars in their regions. The RILs of ICC 4958 X Annigeri have been extensively studied for root traits. An SSR marker (TAA 170) was identified for a major QTL that accounted for 33% of the variation for root weight and 33% of the variation for root length (Chandra 2006).

For efficient and effective germplasm management and conservation, the concept of core and minicore collections have been advocated (Upadhyaya and Ortiz 2001), and trait-specific germplasm has been identified to aid breeding and genomic-assisted selection (Upadhyaya et al. 2012; Meena et al. 2010). Further attempts were also made to characterize the chickpea germplasm at the molecular level in several studies (Iruela et al. 2002; Croser et al. 2003; Nguyen et al. 2004; Rao et al. 2007; Upadhyaya et al. 2008; Sefera et al. 2011; Choudhary et al. 2012a; Sarika et al. 2014) separately from phenotypic characterization (Krishnamurthy et al. 2013a, 2013b; Tapan et al. 2015; Supriya et al. 2017; Neeraj et al. 2016). Characterization of chickpea germplasm for targeted trait-specific germplasm and genomic-assisted selection (Upadhyaya et al. 2012; Roorkiwal et al. 2013) has been reported by numerous researchers. Trait mapping and TILLING approach based on next-generation sequencing (Thudi et al. 2014) have been undertaken to identify genes involved in drought tolerance.

Screening of the chickpea minicore collection for root traits was recently conducted in two different seasons with the double objective of characterizing the genetic variability of drought-avoidance root traits and selecting suitable mapping population parents for molecular mapping of these traits. The complete minicore germplasm collection of *C. arietinum* (211 accessions) along with five popular cultivars as references (216 total entries) were evaluated in PVC cylinders in the first season, along with an additional seven popular cultivars and ten accessions of wild annual species (233 total entries) in the second season. The statistical differences of entries were significant ($P < 0.001$) for both root and shoot traits (Serraj et al. 2004). The root and shoot growth of the wild species was relatively poor compared to *C. arietinum* lines. Some of the germplasm accessions with deep root systems were ICC1356, ICC 3512, ICC4872, ICC13523, and ICC15697. Germplasm accession ICC8261 had the highest root length density and an extremely high root/shoot ratio and rooting depth in both seasons. ICC4958 which is previously the only source used as a deep and large root system parent or control in most drought avoidance studies was confirmed to be an extremely prolific rooting genotype. The new genotypes identified can be used as valuable alternative sources for diversification of mapping populations with varying growth duration and to obtain the required polymorphism for successfully mapping root traits in chickpea.

Accumulation of more superior alleles through marker-assisted recurrent selection (MARS) has also been adopted for enhancing the level of drought tolerance (Varshney et al. 2012) that increases the frequency of numerous desirable alleles having additive effects in recurrent crosses (Bernardo and Charcosset 2006). MARS has proven to be successful in private breeding programs in enhancing genetic gains and is effective at improving quantitative traits in maize (*Zea mays* L.), soybean and sunflower (*Helianthus annuus* L.) (Johnson 2003; Eathington et al. 2007). In brief, MARS is a modern breeding approach that enables us to increase the frequency of

several beneficial alleles with an additive effect and small individual effects in recurrent crosses (Bernardo and Charcosset 2006). Although several multinational companies are using MARS in crops like maize and soybean, only a few public sector institutes have started to use MARS in crops like wheat (Charmet et al. 2001), sorghum (*Sorghum bicolor* (L.) Moench), barley (Abdallah Oukarroum et al. 2009), and rice (Grenier et al. 2012). The use of MARS In chickpea breeding has been reported only at ICRISAT. Four elite "desi" chickpea genotypes were used in pairwise crossing for pyramiding desirable alleles which subsequently led to elite genotypes with enhanced drought tolerance (Thudi et al. 2014). The four superior desi genotypes were selected based on their performance: ICCV 04112, ICCV 05107, ICCV 93954 (released as JG 11 in India), and ICCV 94954 (released as JG 130 in India). Two crosses were made by using elite by elite lines (JG 11 ICCV 04112 and JG 130 ICCV 05107). To pyramid the superior alleles of the favorable QTLs identified based on F3 genotyping data and F5 phenotyping data (from Ethiopia, Kenya and India), a set of eight lines were selected for each cross using OptiMAS ver. 1.0 (Valente et al. 2013). It is anticipated that at the end of the project, RC3F4 progenies will be available for evaluation at multiple locations. These efforts will lead to the development of superior lines with more enhanced drought tolerance. Some efforts have been initiated to use MARS in the case of chickpea for assembling favorable alleles for drought tolerance using ICCV 04112 ICCV 93954 and ICCV 05107 ICCV 94954 crosses. Nevertheless, IARI and IIPR also have initiated MARS in chickpea by using Pusa 372 JG 130 and DCP 92–3 ICCV 10 crosses. These efforts are expected to develop superior lines with enhanced drought tolerance for other ecological regions.

Nevertheless, for understanding the genetics of complex traits like drought tolerance, trait mapping is essential for identifying the genes underlying drought tolerance. Based on the evaluation of the minicore collection for terminal drought tolerance, germplasm lines with prolific root systems were identified, and three recombinant inbred line mapping populations (Annigeri, ICC 4958, ICC 4958 ICC 1882, and ICC 283 ICC 8261) were developed at ICRISAT (Gaur et al. 2008). Comprehensive QTL analysis has provided several stable, consistent, and robust main-effect QTLs for 13 out of 20 drought tolerance traits explaining 10–58.20% of phenotypic variation (Varshney et al. 2014b). Markers flanking these QTLs can be deployed for enhancing drought tolerance as well as individual trait improvement through MABC breeding. A genomic region referred to as "QTL hotspot," spanning ~29 cM on *Cicer arietinum* Linkage Group 04 (CaLG04) of an intraspecific genetic map (ICC 4958 ICC 1882), was found to harbor 12 out of 25 main-effect QTLs for 12 traits explaining ~58.20% of phenotypic variation (Varshney et al. 2014b). Seven SSR markers (ICCM0249, NCPGR127, TAA170, NCPGR21, TR11, GA24, and STMS11) present in QTL hotspot are the most important markers for marker-assisted introgression of this genomic region into elite genetic backgrounds for enhancing drought tolerance through MABC. The data were analyzed for the estimation of genetic components of variance for root traits. These mapping populations are expected to facilitate identification of markers for additional QTLs for root traits.

Similarly, several other mapping populations were also developed for gaining insights into most prevalent biotic and abiotic stresses (Gaur et al. 2014). Next-generation multiparent advanced generation intercross (MAGIC) population is one of a next-generation multiple mapping population comprising of 4–20 parents in cross-combination and a good source of increasing genetic variability. A group of eight chickpea genotypes from different origins was used as parents for the development of a MAGIC population at ICRISAT. Using MAGIC population is beneficial because inclusion of several parents ensures the segregation of QTLs for multiple traits, deployment for understanding complex traits, and the discovery and characterization of novel genes (Glaszmann et al. 2010).

Sequence information and identification of novel genes for agronomically important traits can be done using a number of methods including EST databases (Sreenivasulu et al. 2002). Sanger sequencing and next-generation sequencing techniques have been used for transcriptomic studies of chickpea. Initially EST abundance was assessed for tissue-specific expression, stress-responsive expression, and development-related expression. Chickpea genotypes were grown under drought and salt stresses, and complementary DNA libraries were generated which comprised 20,162 ESTs (Varshney et al. 2009). Another transcriptomic library comprising of 103,215 transcripts (Hiremath et al. 2011) and 53,409 contigs (Garg et al. 2011) have been generated for drought responsiveness. Gene discovery is very limited in chickpea, and few efforts have been made to identify the ESTs associated with stress responses through transcriptomic studies (Varshney et al. 2009). Jain and Chattopadhyay (2010) studied the transcript profiling differences between two chickpea genotypes under different drought treatments and concluded that highly expressing ESTs in tolerant genotypes were encoding proteins involved in transcription, signal transduction, protein metabolism, and cellular organization. Differential downregulation and upregulation of transcriptome has been reported by Deokar et al. (2011) in tolerant and susceptible chickpea genotypes subjected to drought stress. In silico expression studies were also done to know the differential expression of tolerant and susceptible chickpea genotypes under drought stress (Varshney et al. 2009).

Microarray, suppression subtractive hybridization, EST sequencing, and super serial analysis of gene expression (SAGE) have been used for functional genomic analysis of chickpea genotypes in stress-responsive conditions (Buhariwalla et al. 2005; Matsumura et al. 2005, and Molina et al. 2008). The drought- and salinity-responsive transcriptome of chickpea was evaluated using the SuperSAGE technique, reporting that 3000 transcripts were responsive to drought and salinity stresses (Kahl et al. 2007). Transcriptome analysis of chickpea roots was carried out using deep SuperSAGE (combination of next-generation sequencing techniques with SAGE) under normal and water stress conditions, and 17,493 unique transcripts were identified which were drought responsive (Molina et al. 2008). Comprehensive transcriptome analyses demonstrated that osmolyte accumulation, transcription regulation, signal transduction, and ROS scavenging were remodeled under drought stress and were therefore potential target phenomena for improvement of drought tolerance (Molina et al. 2008).

Furthermore, for creating novel alleles and for functional validation of candidate drought-responsive genes, a "target-induced local lesions in genome" (TILLING) population, comprising 10,000 M2 chickpea lines, was also developed by ICRISAT and IARI. A next-generation sequence-based TILLING approach is being adopted to mine novel and potential alleles for some genes associated with terminal drought tolerance (ICRISAT, unpubl. data). Application of marker-assisted selection (MAS) for drought tolerance is still low with little success reported (Oyier 2012; Varshney 2016). The selection based on markers flanking the identified genomic regions in chickpea is expected to accelerate efforts in breeding drought-tolerant varieties. Twenty introgression lines (IL4s) of chickpea harboring the root QTL hotspot from the donor parent ICC 4958 were phenotyped for root and morphological traits under rain-fed conditions. Absence of differences among the ILs for morphological traits indicates similar genetic background of ILs being derived through marker-assisted backcrossing. Marker analysis of the 20 ILs showed presence of the recurrent parent allele in most of the ILs with root QTL.

13.2.1.1 Genomic Selection Approaches for Drought Tolerance

As precise phenotyping is essential and the cost of generating phenotyping data at every generation is very expensive, recent advances in genomics technologies and the availability of a wide range of genotyping platforms have made the cost of genotyping much less expensive compared with phenotyping. Genomic selection is a modern breeding approach that is unlike MABC and MARS; it predicts the breeding values (i.e., the genomic as estimated breeding values) of lines based on historical phenotyping data and the genotyping data. Genomic selection has proven to be successful in several animal breeding programs (Schefers and Weigel 2012; Eggen 2012) as well as in crop plants like maize (Zhao et al. 2012). Efforts to deploy genomic selection in chickpea are underway at ICRISAT. In this regard, a collection of 320 elite breeding lines was selected as the "training population." In addition to compiling historical phenotyping data for ~10 years at >10 locations, research has extensively phenotyped the training population for several traits of agronomic importance at ICRISAT (Patancheru) and IARI (New Delhi) during the cropping season of 2011–2012 and 2012–2013 under rain-fed and irrigated conditions. In parallel, the training population was genotyped using KBioscience Competitive Allele-Specific Polymerase chain reaction (KASPar) assays (651) and diversity array technology (15,360 features). Collected phenotypic data and generated genome-wide marker profiling data (>3000 markers) were used with a range of statistical methods including ridge regression-best linear unbiased prediction, kinship-based ridge regression, BayesCp, BayesB, Bayesian least absolute shrinkage and selection operator (LASSO), and random forest prediction to predict genomic-estimated breeding values (Roorkiwal et al. 2013). Resequencing of the germplasm lines and parents of different mapping populations will enable the identification of genome-wide single-nucleotide polymorphism (SNP) markers that can be effectively utilized in genomic selection.

13.2.1.2 Future Perspectives

As drought is a complex phenomenon, no single approach for all locations may be applicable for enhancing drought tolerance. In this context, an integrated effort deploying need-based approaches is essential. Furthermore, for accelerating the adoption of the molecular breeding for enhanced drought tolerance in chickpea, the development of markers that are easily assayable and technically less demanding, and that do not require high capital equipment for genotyping, termed "breeder-friendly markers," is essential. For instance, conversion of SNPs to Illumina Veracode, cleaved amplified polymorphic sequences or KASPar assays will enable their wider application in breeding programs. In addition, the development of decision support tools is essential for enhancing the precision of selection and to accelerate GAB in crop plants in general. In this area, ICRISAT has developed several important user-friendly decision support tools like the integrated SNP mining and utilization pipeline, the molecular breeding design tool, and the genotyping data management system. Several other tools that aid in genomic-assisted selection have been integrated and made available on an integrated breeding platform (https://www.integratedbreeding.net/molecular-breeding, accessed 6 June 2014). Further well-structured molecular breeding programs are essential for the effective deployment of GAB approaches for crop improvement (Varshney et al. 2013d). To achieve this, training in modern plant breeding skills and fostering integrated breeding strategies and sharing of knowledge and expertise among collaborative partners, especially in developing countries with limited infrastructure and human resources, are the needs of the hour.

13.2.1.3 Candidate Genes for Abiotic Stress Tolerance

Research has revealed several genes are known to be involved in salinity tolerance; the association analysis based on candidate gene sequencing approach is meagerly reported. The salinity-tolerant candidate genes which are supposed to play an important role include *ASR* (abscisic acid stress and ripening gene), *DREB* (drought-responsive element binding proteins), *ERECTA*, *SuSy* (sucrose synthase), *DHN, AKIN, CAD, EREBP, LEA*, and *Myb* transcription factor.

Abscisic acid Stress and Ripening (ASR) gene-ASR is a stress-inducible gene that plays also plays major role in fruit ripening and maturation, has been reported exclusively in plants. Iusem et al. (1993) reported the first Asr gene from cultivated tomato, and since then Asr genes have been found in various species of dicotyledonous and monocotyledonous plants. They also play a vital role in abiotic stress mechanisms like drought, salt, cold, and limited light (Schneider et al. 1997; Huang et al. 2000; Maskin et al. 2001; Jeanneau et al. 2002; Kalifa et al. 2004). Stress endurance through induction of stress-related genes was reported for the dehydration-responsive element binding proteins (*DREB*) transcription factor. Both the forms, *DREB1* and *DREB2*, are reported to be involved in two separate signal transduction pathways under low temperature and dehydration, respectively.

They belong to the ERF (ethylene-responsive element binding factors) family of transcription factors. The roles of DREB proteins in biotic and abiotic stress tolerance were reviewed in detail by Agarwal et al. (2006). *DREB* (Dehydration response element binding) homologue in chickpea was also amplified using primer pairs designed using unigene showing match against *DREB* gene (Srinivasan et al. 1999; Amit et al. 2011). About 1200 bp amplicon for DREB was reported by Roorkiwal et al. (2012).

The *ERECTA* gene codes for a protein kinase receptor, one of a very large and complex family of signaling molecules called protein kinases, and their receptors, which mediates plants responses to disease, predation, and stress. *ERECTA* regulates leaf organogenesis and reduces the density of stomata on the leaf under-surface, hence reduces the evapotranspiration. In *Arabidopsis*, the *ERECTA* gene has been shown to control organ growth and flower development by promoting cell proliferation (Shpak et al. 2004). In *Arabidopsis ERECTA* gene is known to be involved in inflorescence development and organ growth by promoting cell proliferation. Transgenic *Arabidopsis* plants that ectopically overexpress the *ERECTA* gene improve plant transpiration efficiency and drought tolerance by affecting stomatal density, epidermal cell expansion, mesophyll cell proliferation, and cell contact. In addition, Masle et al. (2005) isolated *Arabidopsis ERECTA* gene, a putative leucine-rich repeat receptor-like kinase that regulated transpiration efficiency located on *Arabidopsis* chromosome 2. The *ERECTA* gene can change both leaf stomatal number and leaf structure, and regulate the flowering time, and is proved to regulate plant transpiration efficiency and consequently to have a bright prospect in improving crop drought resistance and using water at high efficiency. The role of the *ERECTA* gene was identified by screening *Arabidopsis* inbred lines and mutant plants, thereby identifying the *ERECTA* homologues in both dicot and monocot crop species. The contribution of *ERECTA* gene toward water use efficiency was confirmed using complementation assays on wilting mutant *Arabidopsis* plants (Masle et al. 2005). The transformation of *ERECTA* gene in the crop species would be major breakthrough in the area of agriculture, with respect to drought tolerance and agronomic performance.

Sucrose synthase *(SuSy) and* sucrose phosphate synthase *(SPS)*—sucrose synthase and the sucrose phosphate synthase are the key enzymes involved in the sugar metabolism pathway. *SuSy* enzyme belongs to the family of glycosyltransferases, especially, hexosyltransferases. It is also commonly known as UDP glucose-fructose glucosyltransferase, sucrose synthase, and uridine diphosphoglucose-fructose glucosyltransferase. The enzyme sucrose synthase (UDP-D- glucose: D-fructose 2a-glucosyltransferase) catalyzes the reversible conversion of sucrose uridine diphosphate into fructose and UDP-glucose. *S-adenosylmethionine synthetase1* gene homologue in chickpea, primers were designed using contig sequence showing similarity against *S-adenosylmethioninesynthetase 1 (SAM1)* gene of *Arabidopsis thaliana*. PCR amplification yielded about 300 bp amplicons across eight chickpea genotypes. Serine/threonine protein kinase (*STPK*) gene homologue was amplified using the STPK specific primer pair designed considering unigene

sequence having similarity with *Arabidopsis thaliana* putative serine/threonine protein kinase. Amplicon size of *STPK* gene approximately 450 bp *STPK* has been shown to play an important role in response to abiotic stress response and seed development in peanut (Rudrabhatla and Rajasekharan 2004).

Although the reaction is reversible, it is thought that the enzyme is mainly involved in the breakdown of sucrose (Huber and Huber 1996; Geigenberger and Stitt 1993; Geigenberger et al. 1995). Hence the activity of sucrose synthase can be important in controlling either starch or cellulose biosynthesis by supplying UDP-glucose as a precursor or as an immediate substrate (Chourey et al. 1991; Delmer and Amor 1995). Ingram et al. (1997) reported the isolation and characterization of cDNA clones encoding SPS from *Craterostigma plantagineum*, *a* resurrection plant in which the accumulation of sucrose is considered to play an important role in tolerance to severe protoplasmic dehydration. It is suggested that the overall regulation of *SPS* is strongly influenced by the changing composition of the cytoplasm in *C. plantagineum* leaves during the dehydration-rehydration cycle.

Researchers have isolated the *AKIN* homologues in various plant species including *Arabidopsis*, rice, potato, and tobacco and established their role in abiotic stress response (Purcell et al. 1998). *AKIN* homologue was amplified using *AKIN* specific primer pair designed considering unigene sequence showing match with *Arabidopsis AKIN* (SNF-1-related protein kinase) with approximate amplicon size of 1100 bp. Amplification of *AMADH* (aminoaldehyde dehydrogenase) homologue yielded a product of ~1200 bp (Roorkiwal and Sharma 2012). Protective/curative role of *AMADH* gene in response to stress events caused by mechanical injury has been reported earlier in pea seedlings (Petrivalsky et al. 2007).

DHNs are one of several proteins that have been specifically associated with qualitative and quantitative changes in cold hardiness (Close 1996). Dehydrin homologue was amplified using primer pair designed for known dehydrin gene using chickpea unigene with approximate amplicon size of 380 bp (Roorkiwal and Sharma 2012).

The role of plant Myb proteins has been well characterized by using different genetic approaches. In most of the cases, the Myb domain binds to a specific DNA sequence (C/TAACG/TG) to facilitate transcriptional activation (Biedenkapp et al. 1988). *Myb* genes were amplified using unigene sequence showing match against *Glycine max Myb* transcription factor (Roorkiwal and Sharma 2012).

Cinnamyl-alcohol dehydrogenase (*CAD*) gene homologue was isolated from eight chickpea genotypes using primers designed for contig showing match with cinnamyl-alcohol dehydrogenase *(CAD)* gene of *Arabidopsis thaliana* (Roorkiwal and Sharma 2012). *CAD* is expected to play a key role in plant defense against various abiotic and biotic stresses (Raes et al. 2003). For isolation of ethylene-responsive element binding protein (*EREBP*) gene homologue in chickpea, primers were designed using contig sequence showing similarity against ethylene-responsive transcription factor from *Arabidopsis thaliana*. Amplification carried out across eight chickpea genotypes produced about 400 bp amplicons (Roorkiwal and Sharma 2012). The *AP2/EREBP* genes play various roles in developmental processes and in stress-related responses in plants.

Late embryogenesis abundant *(LEA)* genes represent a gene family that plays important role in vegetative tissues in response to drought, salinity, cold stress, and exogenous application of abscisic acid (Dure et al. 1989). Primers designed using contig showing sequence similarity with *LEA* domain-containing protein *Arabidopsis thaliana* were used to isolate late embryogenesis abundant *(LEA)* gene in chickpea with approximate amplicon size of 600 bp (Roorkiwal and Sharma 2012).

13.2.2 Salinity

Large land tracts worldwide are being degraded due to salinity, particularly in irrigated areas estimating to about 20 percent (Neeraj et al. 2016). Every day for more than 20 years, an average of 2000 hectares of irrigated land in arid and semiarid areas across 75 countries have been degraded by salt, according to a study by UN University's Canadian-based Institute for Water, Environment and Health (UNU-INWEH 2014). Higher salt concentrations severely affected germination, root and shoot length, and water uptake in chickpea (Neeraj et al. 2016). There is degradation and lysis of germinated root in such salt soils in chickpea. NaCl has more impact than Na_2SO_4. The seeds of chickpea for both landrace have a maximum tolerant level of salinity with 10dS/m. At this salt concentration, significant effect is seen in the seeds compared to the control. But at concentration of 15ds/m of Na_2 SO_4, the germination and growth of seeds are severely affected, and only few seeds start to germinate or raise shoot and root, which dried later (Haileselasie and Teferii 2012).

The tolerance to salinity by chickpea clearly indicated that the sodium to potassium ion concentrations was lower in tolerant lines as compared to sensitive lines. Pod abortion was higher in the salt-sensitive genotypes. However, no effect was seen on pollen viability, in vitro pollen germination, and in vivo pollen tube growth (Turner et al. 2013). The tolerant lines exhibited greater pod number and biomass compared to saline sensitive lines.

Vadez et al. (2007) reported a strong relationship ($r^2 = 0.50$) between the seed yield and salinity. In a study on the seedling parameters in a diverse set of chickpea genotypes under saline stress vs normal conditions, Neeraj et al. 2016 reported maximum reduction in seedling roots weight when germinated in saline conditions. The roots play a major role in establishment of seedling and stem growth, and the highly susceptible lines failed to germinate in saline soils. There was an overall decrease in seedling characters like seedling shoot weight and root biomass. The resistant checks CSG 8962 and JG 62 along with the lines ICCV 00104 and ICCV 06101 showed minimum reduction in seedling characters under salt stress conditions. The yield under saline stress showed a positive correlation with all physiological parameters like RWC and MSI but negative correlation with Na:K ratio under salt stress condition. The traits like higher mean seed yield per plant under saline stress, higher pods per plant, higher RWC, higher MSI, and a low stem Na:K ratio are associated with tolerance to salinity in chickpea. Greater genetic gains can be obtained by using these parameters in selection for salinity tolerance.

Only few studies have reported the presence of QTLs for salinity tolerance with sufficiently large marker interval (Samineni 2016 and Vadez et al. 2012). The nar-

row genetic base in chickpea further limits the efforts to develop stress-tolerant cultivars. The identification of genes associated with salinity stress responses can greatly facilitate the development of improved chickpea cultivars with enhanced salinity tolerance using molecular breeding approaches. The availability of large-scale genomic resources is essential for understanding the biology of complex abiotic stress-like salinity. The availability of next-generation sequencing technologies provides a high-throughput means to study gene expression profiles at the whole-genome level (Garg et al. 2016; Roorkiwal et al. 2014). It has been realized that comparative differential gene expression analysis between genotypes with contrasting response to the stresses can provide a better understanding of the molecular mechanisms underlying tolerance and provide better candidate gene information involved in imparting tolerance to salt stress (Cotsaftis et al. 2011; Lenka et al. 2011). A total 46 QTLs for salinity tolerance was identified using mapping population from ICCV 2 x JG 11. Out of 49 QTLs, 19 QTLs were for phonological traits (7 QTL for days to flowering and 12 QTLs for days to maturity) and 27 QTLs for yield and yield-related traits. Minor QTLs were detected for harvest index (HI) on CaLG04d in salinity treatment, while finding of controlled experiment revealed CaLG07 harbors QTLs for yield, pod number, filled pod number, and seed number (Pushpavalli et al. 2015). QTLs for salinity tolerance are located in the genomic region of CaLG05 flanked by two makers, i.e., CaM0463 and ICCM 272, which contained 17 main QTLs for seven traits (DF, DM, ADM, stem and leaf weight, 100-seed weight, HI, and yield). Genomic region on CaLG07 contains seven QTLs for five different traits, viz., DF, DM, seed number, pod number, and yield. Genomic region on CaLG08 contained eight QTLs for three traits DF, DM, and HI. Out of the abovementioned genomic regions, CaLG05 and CaLG07 genomic regions were most important as they contained QTLs for traits that were remarkably related to yield under salt stress conditions (Puspavalli et al. 2015).

13.2.3 Cold/Chilling

Chilling and freezing are the two categories under the cold stress. The genetic response of genotypes to different stresses appears to be mostly common (Seki et al. 2002). Pollen sterility and flower abortion are the most common effects of cold stress in chickpea when it occurs in the reproductive phase. Tolerance to cold stress becomes all the more important in West Asia and North Africa, Australia, Europe, Canada, and Northern India. Freezing (mean daily temperature < -1.5 °C) and chilling temperatures (mean daily temperature between -1.5 and 15 °C) are known to affect chickpea at various stages of development from germination to maturation (Croser et al. 2003). In these climates and late sown crop of Northern India, chilling temperature in the vegetative stage and cold stress at flowering due to sudden frost greatly reduce the yield of the crop. The breeding procedures focusing on development of cultivars for these regions need to target cold tolerance both at seedling and flowering stages. Screening of germplasm at ICARDA has identified several cold-tolerant lines from the cultivated (Singh et al. 1995) and wild species (Robertson et al. 1995).

Flower abortion due to cold stress at temperatures of 15 °C and below are reported in Australia (Siddique and Sedgeley 1986), the Mediterrranean (Singh 1993), and India (Savithri et al. 1980; Srinivasan et al. 1998). Flower abortion due to cold stress in chickpea is associated with lower levels of sucrose, glucose, and fructose in anthers and pollen (Nayyar et al. 2005). Total sugars and starch were found to be higher in cold-tolerant genotypes compared to the susceptible ones whereas oxidative stress was low (Kumar et al. 2014). Sharma and Nayyar (2014) analyzing a total of 9205 EST bands in cold-tolerant chickpea genotype ICC16349 found that the cold stress altered expression of 127 ESTs (90 upregulated, 37 downregulated) in anthers. Ninety-two of these (two third proportion) were novel with unknown protein identity and function. The remaining about one third (35) belonged to several functional categories such as pollen development, signal transduction, ion transport, transcription, carbohydrate metabolism, translation, energy, and cell division. Limited genes were involved in regulating cold tolerance in chickpea anthers. Moreover, the cold tolerance was manifested by upregulation of majority of the differentially expressed transcripts. The anthers appeared to employ dual cold tolerance mechanism based on their protection from cold by enhancing triacylglycerol and carbohydrate metabolism and maintenance of normal pollen development by regulating pollen development genes. Functional characterization of about two third of the novel genes is needed to have precise understanding of the cold tolerance mechanisms in chickpea anthers (Sharma and Nayyar 2014). Chilling temperatures during early reproductive growth cause yield losses in chickpea in parts of the Indian sub-continent and Australia. The plants continue to produce flowers but fail to set pods when mean daily temperature falls below 15^0 C. ICRISAT scientists have developed several breeding lines (e.g., ICCV 88502, ICCV 88503, ICCV 88506, ICCV 88510, ICCV 88516) that are able to set pods at lower temperature (mean daily temperature between 12and 15 °C). A pollen selection method was developed in Australia and applied to transfer chilling tolerance from ICCV 88516 to chilling sensitive cultivars, leading to development and release of chilling-tolerant cultivars Sonali and Rupali (Clarke and Siddique 2004). RFLP markers for chilling tolerance were identified and subsequently converted to SCAR markers. These were used successfully to select chilling-tolerant progeny from a cross between Amethyst and ICCV 88516 but were ineffective in other crosses (Millan et al. 2006).

13.3 Genomic-Assisted Breeding for Biotic Stress Tolerance in Chickpea

13.3.1 Fusarium *Wilt*

Among the biotic stresses that caused major damage to chickpea production, *Fusarium* wilt caused by *Fusarium oxysporum* f.sp. *ciceris* is the major yield reducer. Losses to the tune of up to 90% have been reported by Singh (1993). Havare

and Neme (1982) have given the race distribution of this pathogen in India. Of the eight races, they identified only IA, 2, 3, and 4 to be prevalent in India. Lines with resistance to this pathogen have been identified and have been used in crop improvement programs. The line WR315 among all the resistant donors is most widely used and has been reported to be resistant to all the races except race 3 (Haware et al. 1997). Molecular markers have been identified for most of the Foc genes (Table 13.1). It has been observed that most of these resistant genes are present in two different linkage groups on different chromosomes, while Teresa Millan et al. (2006) mostly reported it to be present on linkage group 2. Tekeoglu et al. (2000) also reported some of these genes to be present on linkage group 3 too. Improvement of Pusa 256 through marker-assisted backcrossing for introgression of Foc 2 gene using TA37 and TA110 was done by Pratap et al. (2017). Similarly, C214 was improved for resistance against race 1 by Varshney et al. (2014a). Bharadwaj et al. (2011) also reported identification of markers linked to resistance for different races of *Fusarium* wilt and development of introgression lines in a Generation Challenge Project (GCP) along with ICRISAT

13.3.2 Ascochyta *Blight*

The chickpea-growing regions of higher latitudes, i.e., colder regions with cooler, cloudy, and humid weather during reproductive stages, get affected by *Ascochyta* blight (AB). Different workers have reported different pathotypes and subtypes for this disease. Udupa et al. (1998) have reported three pathotypes while Nene and Reddy (1987) reported five pathotypes. Resistant lines have been identified, and some of these like ILC 3279, ILC 195, ILC 482, and ILC 72 developed by ICARDA have been released as varieties. Molecular markers for AB-resistant QTLs and molecular markers linked to them by large number of workers (Table 13.1). The marker-assisted breeding program using the identified QTLs has just been initiated at ICAR-Indian Institute of Plant Research (IIPR), Kanpur; Punjab Agricultural University (PAU), Ludhiana; and ICAR-Indian Agricultural Research Institute (IARI), New Delhi.

13.3.3 Botrytis *Gray Mold*

In the recent past, *Botrytis* gray mold (BGM) is emerging as an important disease of chickpea in the eastern part of Indian subcontinent including Nepal and Bangladesh and North India including Pakistan and Australia (Haware and Mc Donald 1992). This fungus has a very wide host range, and the source of absolute resistance has not been found in *Cicer arietinum* L. germplasm (Pande et al. 2001). Three QTLs were mapped on two linkage groups LG3 and LG6 by Anuradha et al. (2011). Resistance

Table 13.1 List of molecular markers associated with gene/QTLs for resistance to different races of FW and AB

Resistance gene/QTL	Marker	Forward primer sequence (5′- 3′)	Reverse primer sequence (5′- 3′)	Reference
Fusarium wilt resistance				
Foc-0/foc-0	TR59	AAAAGGA ACCTCAAGTGA CA	GA AAATGA GGGA GTGA GA TG	Sharma and Muehlbauer (2007)
foc-1	TA59	ATCTAAAGA GA AATCAAAATTGTCGA A	GCAAATGTGA AGCATGTATAGA TAAAG	Sharma and Muehlbauer (2007)
	TA96	TGTTTTGGA GA AGA GTGA TTC	TGTGCATGCAAATTCTTACT	
	TA27	GA TAAAATCATTATTGGGTGTCCTTT	TTCAAATAATCTTTCATCAGTCAAATG	
foc-2	TA96	TGTTTTGGA GA AGA GTGA TTC	TGTGCATGCAAATTCTTACT	Sharma and Muehlbauer (2007),
	TA27	GA TAAAATCATTATTGGGTGTCCTTT	TTCAAATAATCTTTCATCAGTCAAATG	
	TR19	TCAGTATCACGTGTAATTCGT	CATGA ACATCAAGTTCTCCA	
Foc 1 & 3	GA 16	CACCTCGTACCATGGTTTCTG	TAAATTTCATCCTCTCCGGC	Varshney et al. (2014a)
	TAA60	TCATGCTTGTTGGTTAGCTAGAAA	CAAAGACATAATCGAGTTAAAGAAAA	
	TA194	TTTTTGGCTTATTAGACTGACTT	TTGCCATAAAATACAAAATCC	
	TS82	TCAAGATTGATATTGATTAGATAAAAGC	CTTTATTTACCACTTGCACAACACTAA	
	TA110	ACACTATAGGTATAGGCATTTAGGCAA	TTCTTTATAAATATCAGACCGGAAAGA	
	TR19	TCAGTATCACGTGTAATTCGT	CATGAACATCAAGTTCTCCA	
Foc-3/foc-3	TA96	TGTTTTGGA GA AGA GTGA TTC	TGTGCATGCAAATTCTTACT	Sharma and Muehlbauer (2007)
	TA27	GA TAAAATCATTATTGGGTGTCCTTT	TTCAAATAATCTTTCATCAGTCAAATG	
	TR59	AAAAGGA ACCTCAAGTGA CA	GA AAATGA GGGA GTGA GA TG	
foc-4	TA59	ATCTAAAGA GA AATCAAAATTGTCGA A	GCAAATGTGA AGCATGTATAGA TAAAG	Sharma and Muehlbauer (2007)
	TA96	TGTTTTGGA GA AGA GTGA TTC	TGTGCATGCAAATTCTTACT	
	TA27	GA TAAAATCATTATTGGGTGTCCTTT	TTCAAATAATCTTTCATCAGTCAAATG	
	TR19	TCAGTATCACGTGTAATTCGT	CATGA ACATCAAGTTCTCCA	
	TA194	TTTTTGGCTTATTAGA CTGA CTT	TTGCCATAAAATACAAAATCC	

Foc-5/foc-5	TA27	GA TAAAATCATTATTGGGTGTCCTTT	TTCAAATAATCTTTCATCAGTCAAATG	Sharma and Muehlbauer (2007)
	TA59	ATCTAAAGA GA AATCAAAATTGTCGA A	GCAAATGTGA AGCATGTATAGA TAAAG	
	TA96	TGTTTTGGA GA AGA GTGA TTC	TGTGCATGCAAATTCTTACT	
	TA110	ACACTATAGGTATAGGCATTTAGGCAA	TTCTTTATAAATATCAGA CCGGA AAGA	
	TA59	ATCTAAAGA GA AATCAAAATTGTCGA A	GCAAATGTGA AGCATGTATAGA TAAAG	
	TA53	GGA GA AAATGGTAGTTTAAAGA GTACTAA	AAAAATATGA AGA CTAACTTTGCATTTA	
	TA103	TGA AATATCTAATGTTGCAATTAGGA C	TATGGA TCACATCAAAGA AATAAAAT	
	TS82	CAAAGA CATAATCGA GTTAAAGA AAAA	TGGTTAGCTAGA AAATTCAAGGG	
	TR58	CTCTATATTTGTTTGTTTTTCGTTTTG	TAAAATGTGTAGGGTGCAGA ATAAATA	
Ascochyta blight resistance				
QTL-1	GAA47	CACTCCTCATGCCAACTCCT	AAAATGGA ATAGTCGTATGGGG	Tekeogluet.al. (2000), Flandez-Galvez et al. (2003a)
	TS12b	TTTTTCTTCCCCTTTTCNACAT	ATTCCCTTCCTTYATTWATTTATTTTT	Flandez-Galvez et al. (2003b)
	STMS28	CCCTTCTAGTGA TATTTTG	AAATGTGTTTTATGGA ATAAGTCAT	Tekeoglu et al. (2000), Flandez-Galvez et al. (2003b) Cho et al. (2004)
	STMS11	GTATCTACTTGTAATATTCTCTTCTCT	ATATCATAAACCCCCCAC	
	GA2	TGCATTGGA AATACAGCATGA	AATTTTGGTTCGCCACAAAC	
	TS12b	TTTTTCTTCCCCTTTTCNACAT	ATTCCCTTCCTTYATTWATTTATTTTT	
	TR20	ACCTGCTTGTTTAGCACAAT	CCGCATAGCAATTTATCTTC	
QTL-2	TA3a	AATCTCAAAATTCCCCAAATT	ATCGA GGA GA GA AGA ACCAT	Flandez-Galvez et al. (2003b)
	TA146	CTAAGTTTAATATGTTAGTCCTTAAATTAT	ACGA ACGCAACATTAATTTTATATT	
QTL-2/QTL-3	TA72	GA AAGA TTTAAAAGA TTTTCCACGTTA	TTAGA AGCATATTGTTGGGA TAAGA GT	Flandez-Galvez et al. (2003b)
	GA2	TGCATTGGA AATACAGCATGA	AATTTTGGTTCGCCACAAAC	
	TA3a/ TA3b	AATCTCAAAATTCCCCAAATT	ATCGA GGA GA GA AGA ACCAT	

(continued)

Table 13.1 (continued)

Resistance gene/QTL	Marker	Forward primer sequence (5′- 3′)	Reverse primer sequence (5′- 3′)	Reference
QTL-3	TS45	TGA CACAAAATTGTCTCTTGT	TGTTCTTAACGTAACTAACCTAA	Millan et al. (2003), Iruela et al. (2006), Udupa and Baum (2003)
	TA3b	AATCTCAAAATTCCCCAAATT	ATCGA GGA GA GA AGA ACCAT	
	TA194	TTTTTGGCTTATTAGA CTGA CTT	TTGCCATAAAATACAAAATCC	
	TS82	TCAAGA TTGA TATTGA TTAGA TAAAAGC	CTTTATTTACCACTTGCACAACACTAA	
	TR58	CTCTATATTTGTTTGTTTTTCGTTTTG	TAAAATGTGTAGGGTGCAGA ATAAATA	
AR2	TA72	GA AAGA TTTAAAAGA TTTTCCACGTTA	TTAGA AGCATATTGTTGGGA TAAGA GT	Rakshit et al. (2003)
	TA146	CTAAGTTTAATATGTTAGTCCTTAAATTAT	ACGA ACGCAACATTAATTTTATATT	
ar1	GA16	CACCTCGTACCATGGTTTCTG	TAAATTTCATCCTCTCCGGC	Cho et al. (2004)
ar1a	GA16	CACCTCGTACCATGGTTTCTG	TAAATTTCATCCTCTCCGGC	Udupa and Baum (2003), Cho et al. (2004)
	GA20	TATGCACCACACCTCGTACC	TGA CGGA ATTCGTGA TGTGT	
ar1b	TA37	ACTTACATGA ATTATCTTTCTTGGTCC	CGTATTCAAATAATCTTTCATCAGTCA	Udupa and Baum (2003)
	TA200	TTTCTCCTCTACTATTATGA TCACCAG	TTGA GA GGGTTAGA ACTCATTATGTTT	
ar2a	GA16	CACCTCGTACCATGGTTTCTG	TAAATTTCATCCTCTCCGGC	Cho et al. (2004)
	GA24	TTGCCAAAACCAATAACTCTG	TCCCTTTTACACAAGGCCAG	
	GAA47	CACTCCTCATGCCAACTCCT	AAAATGGA ATAGTCGTATGGGG	
ar2b	TA130	CACTCCTCATGCCAACTCCT	AAAATGGA ATAGTCGTATGGGG	Collard et al. (2003)
	TA72	GA AAGA TTTAAAAGA TTTTCCACGTTA	TTAGA AGCATATTGTTGGGA TAAGA GT	
	TS72	CAAACAATCACTAAAAGTATTTGCTCT	AAAAATTGA TGGA CAAGTGTTATTATG	
Ar19	TR19	TCAGTATCACGTGTAATTCGT	CATGA ACATCAAGTTCTCCA	Collard et al. (2003)
	GA16	CACCTCGTACCATGGTTTCTG	TAAATTTCATCCTCTCCGGC	

to this pathogen has been identified in wild *Cicer* sp. *Cicer bijugum* (Haware et al. 1992). Punjab Agricultural University (PAU), Ludhiana, has developed some lines through pre-breeding having tolerance/resistance to *Botrytis* gray mold. Comprehensive evaluation of these lines, their derivatives, and previously reported sources in field and in controlled conditions for their level of tolerance/resistance is being done by ICAR-Indian Institute of Plant Research (IIPR), Kanpur; Punjab Agricultural University (PAU), Ludhiana; ICAR-Indian Agricultural Research Institute (IARI), New Delhi; and G.B. Pant University of Agriculture and Technology, GBPUA&T (Pantnagar).

13.4 Molecular Markers

The most recent quindecennial (2002–2017) has seen the advancement of substantial level of genomic assets in chickpea. Simple sequence repeats (SSR) markers, most favored markers for molecular breeding, were accessible in exceptionally predetermined number in this crop until 2005. Paucity of polymorphic molecular markers in chickpea (*Cicer arietinum* L.) has been a major limitation in the improvement of this important legume. However it is not so anymore. The concerted efforts by chickpea workers and generous funding and efforts by Indian Council of Agricultural Research (ICAR), Generation Challenge Programme, The Bill and Melinda Gates Foundation, Department of Biotechnology (DBT), etc. have led to the development of large-scale molecular markers, construction of comprehensive linkage map, and draft genome sequencing. ICRISAT, NIPGR, and NRCPB have been in forefront in the development of marker repertoire (Sethy et al. 2006, Varshney et al. BMC Genomics 2009). 2000 genomic SSR markers chickpea have been developed (Varshney et al. 2007; Nayak et al. 2010; Thudi 2011), ESTs (Varshney et al. 2009), 454/FLX transcript reads (Hiremath et al. 2011; Garg et al. 2011,) and BAC-end sequences (Thudi et al. 2011). 26,082 potential SNPs have been identified (Hiremath et al. 2011) based on alignment of ~37 million Illumina/Solexa tags. Similarly, at National Institute of Plant Genome Research (NIPGR), a set of 487 novel functional markers including 125 EST-SSRs, 151 intron-targeted primers (ITPs), 109 expressed sequence tag polymorphisms (ESTPs), and 102 SNP markers has been developed (Choudhary et al. 2012b). Though DArT markers were developed in pigeon pea, their use was mostly restricted to introgression studies as these were very less polymorphic in the cultivated pigeon pea (Thudi et al. 2011). KASPar assays for 2005 SNPs in chickpea (Hiremath et al. 2012) were developed. High throughput SNP genotyping platform utilizing DArT and next generation sequencing (NGS) technology like pyrosequencing (Alderborn et al. 2000; Ching and Rafalski 2002; Varshney et al. 2009), mass spectrum analysis (Rodi et al. 2002), Affymetrix chip (Borevitz et al. 2003), Golden Gate assay (Fan et al. 2003; Rostoks et al. 2006), Roche 454/FLX, AB Biosystem, and Illumina/Solexa are used for whole-genome transcription identification techniques to spot genomic regions and genes underlying plant

Table 13.2 Molecular markers in chickpea (Advances in Pulse Research, IIPR 2012)

Marker	Number of markers	Reference
Genomic SSR	2328	Hüttel et al. (1999), Winter et al. (1999), Sethy et al. (2006), Lichtenzveig et al. (2005), Choudhary et al. (2006), Eujayl et al. (2004), Sethy et al. (2006), Qadir et al. (2007), Nayak et al. (2010), ICRISAT-UC Davis, USA
EST-SSR	508	Choudhary et al. (2009), Varshney et al. (2009b), Gaur et al. (2011)
CAPS	306	Rajesh and Muehlbauer (2008), Varshney et al. (2007), Varshney et al. (2009a), Gujaria et al. (2011)
DArT	15,360	DArT Pvt. Ltd, Australia And ICRISAT
SNPs	Ca. 9000 identified and 768 on Golden Gate assay 1893	ICRISAT, UC-Davis, USA and NCGR, USA

stress responses (Varshney et al. 2009; Varshney et al. 2010) to develop massive scale SNPs and using for genotyping to develop highly saturated genetic and transcript maps (Gujaria et al. 2011). Approximately 15300 (by DArT Pvt. Ltd, Australia And ICRISAT)DArT are available in chickpea featuring 21500 array, 300 panel resulted in 5400 polymorphic features and ~200 maker loci on genetic map (Varshney et al. 2010) (Table 13.2).

13.5 Conclusion

Advances in sequencing and genotyping technologies helped in generation of several thousand markers including SSRs, SNPs, and DArTs and hundreds of thousands transcript reads and BAC-end sequences in chickpea. Comprehensive transcriptome assemblies and genome sequences have either been developed or underway for other important traits including quality, herbicide tolerance, salinity, etc. Based on these resources, dense genetic maps, QTL maps as well as physical maps for chickpea have also been developed. As a result, chickpea graduated from "orphan" or "less-studied" crops to "genomic resource-rich" crops. Genomic-assisted breeding approaches in the form of marker-assisted selection (MAS) and marker-assisted backcrossing (MABC) for introgressing QTL region for drought tolerance-related traits; *Fusarium* wilt resistance and *Ascochyta* blight resistance in chickpea have also been initiated. However, it is critical to use other modern breeding approaches like marker-assisted recurrent selection (MARS), advanced-backcross (AB-backcross) breeding, and genomic selection (GS) to utilize the full potential of genomic-assisted breeding for crop improvement.

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Chapter 14
Genomic-Assisted Breeding in Oilseed Brassicas

Surinder K. Sandhu and Gurpreet Singh

Abstract The oilseed brassicas, world's third most important source of vegetable oil with recently gained interest as a source of biodiesel, occupy a prominent place in the world's agrarian economy and are grown in more than 50 countries across the globe. Improvement in nutritional profiling of *Brassica* oil and its defatted oil cake has vastly spread the production domain of rapeseed-mustard in the world. Consistent breeding efforts led to conversion of almost all *Brassica napus* into present-day canola-quality cultivars, and intensification of this quality trait in *Brassica juncea* too is leading its expansion in drier and low rainfall areas of the world. The good agronomic performance and the energetic balance of *Brassica carinata* in semiarid temperate climate and under low cropping system have generated a new interest in this species as an oilseed crop. Though, a young species with a short domestication history, *Brassica napus* has gained a huge attention of researchers and consequently, has witnessed a steady progress during last four decades. The conventional breeding as well as modern biotechnological tools has led to the improvement of various agronomically important quantitative and qualitative characters in oilseed brassicas.

Arabidopsis, the closest relatives of *Brassica* species, besides evolutionary divergence, offers great potential for genetic and physical comparative mapping to identify genomic regions harboring genes of interest and to accelerate marker development, map-based gene cloning, and candidate gene identification in *Brassica* crops. Multinational Brassica Genome Project, initiated in January 2003, has given great impetus to the Brassica genomic research, and thereafter, availability of genome sequence information has allowed the construction of high-resolution genetic maps, delineating QTLs underlying complex quantitative economic traits and their conversion in perfect markers, and to tag genes of commercial interest. In spite of the difficulties in QTL localization in these polyploid crops, trait-associated genetic markers have been identified for yield component traits, fatty acid

S. K. Sandhu (✉)
Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana, India
e-mail: surindersandhu@pau.edu

G. Singh
PAU-Regional Research Station, Faridkot, Punjab, India

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composition controlling domains and for a couple of biotic and abiotic stresses for applications in *Brassica* molecular breeding. Though, consistent improvement for productivity, oil content, oil quality and tolerance to biotic and abiotic stresses in Oilseed brassicas has been achieved but synchronous maturity, stable and practically viable male sterility systems for hybrid development, shattering resistance in *B. napus*, defined and efficient DH production systems, and harnessing the potential as biodiesel crops are upcoming areas of research through combination of traditional and genomic approaches.

Keywords Genomic- assisted breeding · Molecular breeding · Molecular- assisted selection · Oilseed brassicas · Biotic stresses · Abiotic stresses

14.1 Introduction

The genus *Brassica* which belongs to angiosperm family Brassicaceae, commonly known as Cruciferae because of the characteristic Greek cross-shaped four-petal flowers, includes many economically important vegetable, condiment, forage, and oilseed crops. The word, Brassica is originated from the *Bresic* or *Bresych*, the Celtic name for cabbage (Hegi 1919). A distinctive feature of the tribe Brassiceae is extensive subsequent genome triplication, an indicative of a hexaploidy event. After a split into separate *Arabidopsis* and *Brassica* lineages ~20 MYA (Yang et al. 1999), *Brassica* underwent a whole-genome triplication event, followed by extensive diploidization (Chalhoub et al. 2014a). Several comparative mapping studies between *Brassica* and *Arabidopsis thaliana* had revealed the triplicate nature of the *Brassica* genome, with an average of three copies of each chromosomal segment of *A. thaliana*, which resulted from the triplication of the whole Brassica genome at 11–12 MYA (Mun et al. 2009). The resulting mesohexaploid *Brassica* genomes (*B. rapa* (AA), *B. nigra* (BB) and *B. oleracea* (CC)) are predicted to encode up to three orthologs of each *Arabidopsis* gene.

Economically important species belonging to *Brassica* genus include three diploid species, viz., *B. rapa* (AA, $n = 10$), *B. nigra* (BB, $n = 8$), and *B. oleracea* (CC, $n = 9$), and three derived allotetraploid species, viz., *B. juncea* (AABB, $n = 18$), *B. napus* (AACC, $n = 19$), and *B. carinata* (BBCC, $n = 17$) (UN 1935). The genome relationship of three monogenomic species and three digenomic species is well known as U's triangle (Fig. 14.1). It is also now known that *B. nigra*, *B. rapa*, and *B. oleracea*/*B. rapa* are the cytoplasm donor species for *B. carinata*, *B. juncea*, and *B. napus*, respectively (Banga et al. 1983). The oilseed brassicas comprise four major species, namely, *B. rapa*, *B. juncea*, *B. napus*, *and B. carinata. Brassica napus* L. spp. *oleifera*, $2n = 4 \times = 38$, genome $A^nA^nC^nC^n$, is originated along the Mediterranean coastline at least 10,000 years ago as a result of spontaneous hybridization between turnip rape (*Brassica rapa* L.; genome A^rA^r, $2n = 2 \times = 20$) and cabbage (*Brassica oleracea* L.; genome C^oC^o, $2n = 2 \times = 18$), followed by chromosome doubling (UN 1935; Gomez-Campo and Prakash 1999). Based on chloroplast and nuclear markers diversity, the original hybridization event resulting into *B. napus* occurred on more than one occasion and involved different maternal

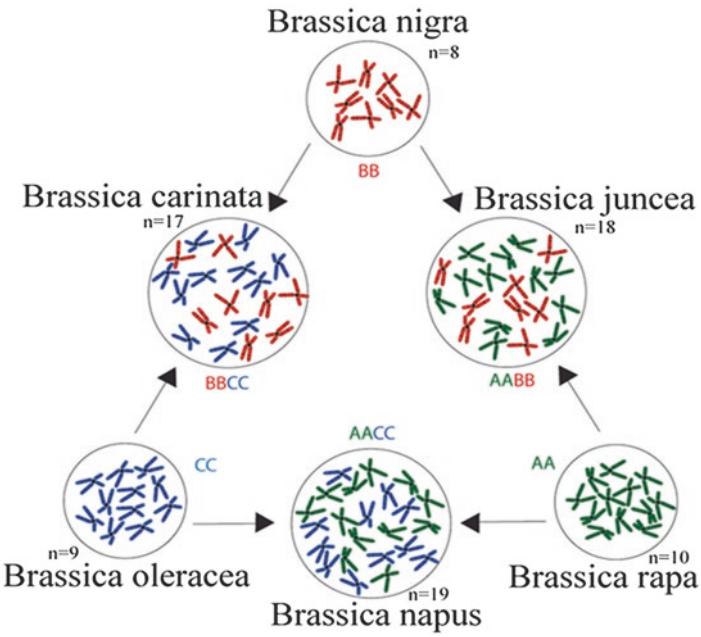

Fig. 14.1 The triangle of U depicts the genomic relationship of six *Brassica* species. Each letter n indicates the number of chromosomes found in the gametes of each species. Diploid species are found in the corners, which hybridized with one another to create the respective alloploid species found in between. (Adapted from the Wikimedia Commons file "Image: Triangle of U Simple. PNG" http://commons.wikimedia.org/wiki/File:Triangle of U Simple.PN)

genotypes (Allender and King 2010). It is considered as a young species because of a short domestication history spanning only 400–500 years (Gómez-Campo 1999). It can hybridize with *B. rapa*, *B. oleracea*, *B. juncea*, and *B. carinata* as they share at least one common genome. Several studies have been published on resynthesized *B. napus* revealing meiosis-driven genome reshuffling in allopolyploids (Szadkowski et al. 2010). It has four morphotypes, viz., oilseed rape (canola), rutabaga, fodder rape, and kale morphotypes grown for oil, fodder, and food, which were the result of diversifying selection over a long period of time (Chalhoub et al. 2014b). Indian mustard (*Brassica juncea*) is a major oilseed crop of Indian subcontinent, northern China, and east European countries. It is also an option for stressed ecologies of Canada and Australia. *B. juncea* (AABB; $2n = 36$) is an allotetraploid that arose several times through independent hybridization events between *B. rapa* (AA; $2n = 20$) and *B. nigra* (BB; $2n = 16$) in the areas encompassing Mediterranean, Irano-Turanian, and Saharo-Sindian geographies (Kaur et al. 2014). It is an important winter season oilseed crop in India and contributes nearly 28.6% of edible oil supplies (GOI 2017). *B. juncea* is also cultivated in China and Eastern Europe as oilseed, vegetable, or condiment crop. With the development of canola-quality *B. juncea*, it was further expansed to drier regions in Australia and Canada (Parker 1999). Mustards were naturalized to Australia and New Zealand, and Australia

emerged as the major exporter of Brassicaceae oilseeds to meet the global demand for healthy plant-derived oil having high content of polyunsaturated fatty acids (Burton et al. 2003). Canola-quality Indian mustard (*Brassica juncea*) was developed as a complimentary oilseed crop to canola (*B. napus*) for cultivation in hot and low rainfall areas (Oram and Kirk 1995), where canola did not perform well. Moreover, *B. juncea crop is* not only superior to *B. napus* with respect to heat and drought tolerance and seed shattering but also infused with resistance to blackleg disease, one of the most detrimental diseases in *B. napus* (Woods et al. 1991).

B. carinata, a native plant of the Ethiopian highlands, has drawn increased interest due to its better agronomic performances in areas such as Spain, California, and Italy, characterized by unfavorable environmental conditions for the cultivation of *B. napus* (by far the most common rapeseed cultivated in continental Europe) (Cardone et al. 2003). The agronomic performance and the energetic balance confirmed that *B. carinata* adapted better and was more productive both in adverse conditions (clay- and sandy-type soils and in semiarid temperate climate) and under low cropping system when compared with *B. napus* (Cardone et al. 2003).

Brassica rapa L. (syn. *B. campestris*), a diploid species ($2n = 20$, AA), is an important *Brassica* species that is grown widely to produce leafy vegetables in Korea, China, and Japan, for vegetable oil in India, China, and Canada, and as a fodder crop in Europe. Chinese cabbage, *pak choi*, *sarson*, and turnips are distinct morphotypes of *B. rapa* belonging to different subspecies that are adapted to different geographical regions and climatic conditions. It has been suggested that *B. rapa* appeared from wild plants belonging to the $n = 9$ cytodeme of *B. oleracea* as the result of the addition of one chromosome and some chromosomal rearrangements (Song et al. 1990; Warwick and Black 1991). The spread of this species into East (India, China, and Japan) certainly conveyed the idea of its North-East Mediterranean origin (Ignatov et al. 2008). The Brassicaceae also includes the most intensively studied model plant, *Arabidopsis thaliana*. In molecular genetic studies of Brassicaceae crops, the genomic information on *A. thaliana* proved highly useful, and led to identification of many genes of economic importance.

14.2 Breeding Objectives

Crop improvement is a continuous pursuit of greater productivity and excellent genetic gains have been achieved in the past in oilseed brassicas. The major breeding objectives in oilseeds brassicas are higher productivity coupled with quality, resistance to various biotic stresses and with current emphasis on determinate plant type for acquiescence to mechanical operations, shattering resistance in *B. napus*, and tolerance to abiotic stresses for acclimatization to climate changes. The development of canola-quality Indian mustard (*B. juncea*) has also being taken up as breeding objective for its suitability in hot and low rainfall areas as a complimentary oilseed crop to canola (*B. napus*). The choice of varieties, available genetic variability, and mode of reproduction determine the methods of breeding. In *B. rapa*, a

cross-pollinated crop determined by saprophytic self-incompatibility, the choice of development of cultivars is composites, synthetics, open-pollinating populations, and hybrids whereas development of pure-line varieties intended in self-pollinated *B. napus*, *B. juncea*, *and B. carinata*. The development of commercial hybrids is also gaining importance with the availability of cytoplasmic male sterility fertility restoration systems. With the documentation of yield heterosis of up to 150% and with availability of genetic emasculation and effective pollen shedders (Banga 1993), the potential of hybrid development in oilseed brassica is emerging as great opportunity. Seed yield, with major component traits, viz., siliques per plant, seeds per silique, and seed weight (Chen et al. 2007), is the end product of many biological processes, and their expression is controlled by a wide range of complex polygenic systems. Yield is also indirectly influenced by other yield-related traits, such as plant height, branch number, and resistance to biotic and abiotic stresses. Hence, it is difficult to accurately evaluate and select for high-yield traits in conventional breeding programs, owing to the influence of the interactions between the environment and the genotype in all growth and development processes (Quarrie et al. 2006). Quantitative traits are affected by several genes of either large or small effect or a combination of both (Falconer and Mackay 1996). Polygenic traits tend to be approximately normally distributed in a population as the alleles affecting the trait can be of similar or conflicting effects and are disseminated differently among individuals and, hence, expressed in a continuous range. Moreover, genotype x environmental interactions reduces the precision of selection by breeder. Visual selection and selection based on single-plant yield in early segregating generations are highly ineffective. Dual bottlenecks of polyploidy and domestication also led to a very narrow genetic base in amphiploid brassicas (Jain et al. 1994) and consequently, a slackening response to selection (Chauhan et al. 2011). The application of molecular marker techniques for quantitative trait locus (QTL) analysis has proved to be a powerful genetic approach to dissect complex traits (Paran and Zamir 2003), though map-based cloning of quantitative trait loci (QTLs) in polyploidy crop species remains a challenge due to the complexity of their genome structures. The complexity of polyploid genomes results in difficulties in QTL localization such as the inaccuracies caused by one homologous sequence from different chromosomes and the interactions between homolog genes (Liu et al. 2015).

14.3 Molecular-Assisted Selection (MAS)

Marker-assisted selection (MAS) techniques have emerged as highly useful tool in accelerating economic breeding programs, which enables identification of the presence of a specific gene or combination of genes that carry a desirable trait, such as insect and disease resistance, or genes affecting agronomic traits and yield through the use of various genetic assays. Molecular markers are very effective in selecting desirable traits which are governed by multiple genes and are not easily assessable through conventional breeding approaches. This technology helps in efficient selection at early stage of new genotypes and faster delivery of products to growers.

14.3.1 Molecular-Assisted Breeding for Flowering Time

Brassica oilseed crops are grown as winter season crops in tropical or subtropical regions. In these regions, high temperature during terminal stage of the crop leads to significant yield loss and, therefore, early flowering and, hence, early maturing cultivars generally express higher productivity (Singh et al. 2014). Shi et al. 2009 documented highly positive association of seed yield with early flowering in vernalization-responsive *B. napus.* Days to flowering have been correlated well with days to maturity in both B. *napus* (Honsdorf et al. 2010) and in *B. juncea* (Mahmood 2007) and, hence, emerged as key trait in breeding for early maturing genotypes. It is difficult to follow conventional breeding methods as flowering time is a complex trait, controlled by a large number of loci and their interactions (Long et al. 2007). Genes involved in different pathways, such as photoperiod, vernalization, and autonomous (flowering independent of environmental cues), and their interactions regulate flowering time trait; hence molecular-assisted breeding could be of great value to breed for this trait. Therefore, identification of photoinsensitivity or flowering controlling gene(s) under short-day conditions will be of enormous prospective to develop early maturing high-yielding brassica cultivars. Out of 20 QTLs associated with flowering time and grain yield variation, two QTLs located on chromosomes A07 and C03 were detected repeatedly across experiments. A negative correlation between flowering time and grain yield was established, and 22 putative candidate genes had been reported for flowering time as well as grain yield located in range of 935 bp to 2.97 Mb from markers underlying QTLs (Raman et al. 2016). In vernalization pathway, a key regulator gene *FLOWERING LOCUS C* (*FLC*) represses the expression of the floral integrator genes *FT*, *FD*, and *SOC1* leading to failure of flowering (Rahman et al. 2018). Under vernalization or low-temperature conditions, *FLC gene gets* downregulated, and hence, floral integrator genes promote flowering. The *FLC* is activated by its upstream regulator *FRIGIDA* (*FRI*) gene. *FRI* and *FLC genes are* major determinants for natural variation of flowering in response to vernalization or low temperature. Rahman et al. (2017) introgressed earliness of flowering from *B. oleracea* into spring *B. napus* canola through interspecific crossing. Based on QTL mapping studies, using double haploid population and publicly available as well as designed markers based on flowering time genes, five genomic regions associated with days to flower on C1, C2, C3, and C6 have been reported and out of these the single QTL of C1 was detected in all trials. Later, Rahman et al. (2018) detected a single QTL on C9 at 10 h photoperiod condition which found to exert an additive effect as well as to show significant interaction with the C1 QTL, which could be detected in all environments and affect days to flowering apparently through the autonomous pathway. The genomic region of C9 might carry a gene affecting flowering time where the allele introgressed from *B. oleracea* var. *alboglabra* is capable of enhancing flowering at 10 h photoperiod condition either directly or in interaction with the gene located on C1. The molecular markers and the genomic regions identified could potentially be used in breeding for the development of early flowering photoinsensitive *B. napus* canola cultivars, as well as for identification of candidate genes involved in flowering time variation and photosensitivity.

14.3.2 Molecular-Assisted Breeding (MAS) for Seed Coat Color

The seed coat color of oilseed brassicas is divided into two major categories, brown/black and yellow. Due to thinner seed coat, oil content of yellow seed found to be 6% higher than that of brown seed (Stringam et al. 1974). A more transparent oil, lower-fiber content, and higher-protein content are other advantages of yellow seeds (Getinet et al. 1996). The oil cake of yellow-seeded cultivars with lower hull proportion also improves the feed value for poultry and livestock (Tang et al. 1997). Rahman et al. (2007) indicated that seed coat color was determined by dominant epistatic gene interactions where the brown color was dominant over the yellow seed coat color. Brown seed coat color controlled by a single dominant gene has been well documented (Chen and Heneen 1992; Chen and Pikaard 1997; Zhang et al. 2009; Xiao et al. 2012). Heneen and Jørgensen (2001) developed a RAPD marker for the seed coat color trait linked to chromosome four of *B. alboglabra*. Somers et al. (2001) identified eight RAPD markers co-segregating with the major yellow seed coat color gene (pigment1) in *B. napus*. Liu et al. (2005) reported two RAPD and eight AFLP markers linked to the seed coat color genes in *B. napus*. Out of ten, two were very close markers to the seed coat color gene (3.9 cM and 2.4 cM) and allowed selection accuracy of 99.91% for yellow-seeded individuals. In *B. juncea*, three tightly linked AFLP markers to brown seed color trait were developed by Negi et al. (2000). Three microsatellite markers (Ra2-A11, Na10-A08, and Ni4-F11) strongly associated with seed coat color genes were developed by Padmaja et al. (2005).

14.3.3 Molecular-Assisted Breeding for Pod Shattering

Worldwide, pod shattering in *B. napus* is a major problem for commercial rapeseed production, as it may lead up to 50% yield loss, and no source of shattering resistance is available in world germplasm of *B. napus*. It is believed that unlike the key cereal species, during the domestication of rapeseed, total prevention of pod shattering and consequent, seed loss has not been targeted for selection (Raman et al. 2011). Earlier efforts were made for introgression of pod shattering resistance in *B. napus from B. juncea* (Prakash and Chopra 1988). In spite of evolutionary divergence, the development and pod structure of *B. napus* were found to be very similar to that of *Arabidopsis*, and through genome-wide association studies, a large number of loci, including those that are involved in shattering in *Arabidopsis*, account for variation in shatter resistance in diverse *B. napus* germplasm (Raman et al. 2011). In this study, 192 accessions of diverse set of *B. napus* were genotyped with 1513 markers (DArT, SSRs, and two candidate genes from *Arabidopsis thaliana*) and phenotyped by recording rupture energy through pendulum test and identified 150 associations for shatter resistance through association mapping. Study reported significant variation for rupture energy that could provide resistance to shattering resistance. Limited genetic diversity for shatter resistance genes in *B. napus* led to

inference that either many of the genes controlling this trait were not included during the natural creation of this species or were not retained during the domestication and selection process (Raman et al. 2011).

14.3.4 Molecular-Assisted Breeding for Seed Yield and its Component Traits

In rapeseed, seed yield was found to be directly determined by yield-related component traits, viz., thousand seed weight, number of pods per plant, and number of seeds per pod (Quarrie et al. 2006). Li et al. (2007) also reported the indirect influence of other traits on seed yield such as biomass yield, plant height, first effective branch height, first effective branch number, length of main inflorescence, and number of pods on main inflorescence. The productivity traits were primarily quantitative and are controlled by nuclear genes. Primacy of additive genetic variation with some dominance and occasional epistasis was emphasized (Banuelos et al. 2013). QTL analysis has proved a powerful genetic approach to dissect complex traits (Paran and Zamir 2003). QTL identification for yield and various yield component traits have been conducted in many different types of populations using various mapping strategies. Extensive research has been documented in *B. napus*, and several groups have associated QTLs with yield and yield-related traits in oilseed brassicas (reviewed by Snowdon and Friedt 2004). The further findings on this aspect have been updated in this chapter (given in Table 14.1). Li et al. (2007) reported 133 QTLs for 12 yield-related traits, out of which 14 showed consistent associations across 2 locations. Eight of 10 QTLs for yield per plant were found to be associated with number of seeds per silique (SS), number of siliques per plant (SP), and 1000 seed weight (SW). In addition, 45 functional markers involved in 39 expressed sequence tags (ESTs) were linked to the QTLs of 12 traits. A high-density linkage map was constructed in *B. napus* using 786 markers covering 19 chromosomes with average distance of 2.7 cM between markers and through meta-analysis, 47 consensus QTLs relevant for seed yield have been documented (Shi et al. 2009). Seed yield was negatively correlated with flowering and maturity times while exhibited positive correlation with other traits. Three yield component traits, viz., pod number, seed number, and seed weight, were negatively correlated in pair-wise comparisons suggesting competition among the sinks for assimilates. Using the large DH population and two corresponding sets of backcrossed test hybrids of *B. napus*, Basunanda et al. 2010 identified co-localized QTL from both populations which could harbor genes involved in regulation of heterosis (including fixed heterosis) for different traits throughout the plant life cycle, including a significant overall influence on heterosis for seed yield. Heritability estimates in multiple environments revealed moderate to high heritability for plant height, silique traits, and yield. Through comparative mapping among *Arabidopsis* and *Brassica* species, 12 candidate genes underlying eight QTLs for seed weight were identified, and a gene-specific marker for BnAP2 was developed through molecular cloning using a seed weight/size gene

Table 14.1 Selected examples of the some of important traits for which genetic marker techniques have been used to map loci or clone gene(s) in *Brassica* oilseeds

Character	Specific trait	Selected references
Disease resistance	Blackleg (*Leptosphaeria maculans*)	Dion et al. (1995), Ferreira et al. (1995a), Pilet et al. (2001), Christianson et al. (2006), Larkan et al. (2013, 2015)
	White rust (*Albugo candida*)	Ferreira et al. (1995b), Kole et al. (1996), Kole et al. (2002b), Panjabi et al. (2010), Singh et al. (2015)
	Turnip mosaic virus (TuMV)	Walsh et al. (1999), Jiang et al. (2010), Lydiate et al. (2014)
	Turnip yellow virus (TuYV)	Dreyer et al. (2001)
	Sclerotinia sclerotiorum	Zhao and Meng (2003)
Oil content/quality	Erucic acid content	Ecke et al. (1995), Das et al. (2002), Saini et al. (2016)
	Glucosinolate content	Uzunova et al. (1995), Howell et al. (2003), Pushpa et al. (2016)
	Oleic acid and linoleic acid content	Hu et al. (1999, 2006)
	Linolenic acid content	Hu et al. (1995), Tanhuanpaa et al. (1995), Jourdren et al. (1996), Uzunova et al. (1995), Howell et al. (2003)
Abiotic stress	Cold tolerance, winter hardiness	Kole et al. (2002a)
	Phosphorus stress	Ren et al. (2012)
	Boron deficiency	Sun et al. (2012)
Male sterility	"Ogura" fertility restorer	Delourme et al. (1998), Brown et al. (2003)
	Polima' fertility restorer	Jean et al. (1998)
	Kosena' fertility restorer	Imai et al. (2003)
	Tournefortii fertility restorer	Janeja et al. (2003)
	BnMs1 and BnMs2 genes, fertility restorer	Yi et al. (2010)
Morphological traits	Plant height (dwarf locus)	Foisset et al. (1995), Barret et al. (1998)
	Flowering time	Ferreira et al. (1995c)
	Petal-less flower	Fray et al. (1997)
	Seed color	Somers et al. (2001)
	Seed coat color	Wei et al. (2007), Xu et al. (2007), Ni et al. (2008), Akhov et al. (2009), Auger et al. (2009), Chai et al. (2009), Zhang et al. (2009), Qu et al. (2016)
	Multi-locule	Xiao et al. (2013), Xu et al. (2017)
	Pod dehiscence	Tan et al. (2009), Tao et al. (2017)
	Branch angle	Wang et al. (2016a)
	Number of seeds per silique	Zhang et al. (2012), Li et al. (2015)
	Seed yield	Chen et al. (2011), Li et al. (2013)
	Drought adaptation	Fletcher et al. (2016)
Pollination method	Cleistogamy	Lu et al. (2012)

Modified from Snowdon and Friedt (2004)

distribution map in *B. napus* (Cai et al. 2012). Thirteen consensus QTLs for seed weight and nine for silique length in *Brassica napus* through preliminary linkage mapping were identified (Li et al. 2014a). The pleiotropic effects for the genetic basis of the three pairs of co-localized seed weight and silique length QTLs have also been highlighted (Li et al. 2014b). Association analysis on a diversity fixed foundation set of 48 inbred lines with 158 SSR markers for root traits and grain yield components under irrigated and water-limited environments shown 13 significant associations between molecular markers and agronomic traits (Akhatar and Banga 2015). Out of which, four markers with grain yield, three with seed size, two with secondary branches, and one marker each with plant height, root diameter, and root length were found to be associated. Identification of grain yield-related QTLs on chromosomes A2 and A3 of the A genome validated the QTLs reported by Zhou et al. (2014) in *B. rapa*, the A genome donor for *B. juncea*. The grain yields QTLs reported for chromosomes 2 and 3 of the B genome were novel.

Breeding efforts toward plant root system modifications are inadequate due to the complexity of this trait and difficulties associated with large-scale nondestructive analysis on root traits during plant growth. But nowadays, more interest is being laid to study root traits due to the association with drought tolerance and grain yield potential (Qu et al. 2008). A deep root system, in place before the onset of drought, with thick roots and an extensive branching ability is always a major component of drought avoidance in mustard (Akhtar and Banga 2015). Only two root traits, root area and root length, were discovered to be linked with markers NI2-E12-3 [−log10(P) = 3.335] and SB5162 [−log10(− P) = 3.363], respectively, and could be of high importance in Indian mustard breeding program as this is a crop of rain-fed ecologies. Colocated genes were reported for seed weight and silique length in a major QTL identified on chromosome A9 of *B. napus* (Liu et al. 2015). Reciprocal crossing study revealed that the QTL affects seed weight by maternal effects. A major pleiotropic locus/QTL which may lead to compact plant architecture by reducing plant height, changing leaf shape, and altering plant type traits was also identified (Wang et al. 2016a). Several yield and yield-related traits such as 1000-seed weight, number of seeds per silique, silique size, silique length, and resistance to bacterial leaf blight, shattering, and lodging were found to be colocated in the same genomic regions further emphasizing pleiotropic effects for those regions (Wang et al. 2016b). Trait-by-trait meta-analysis revealed that the 144 consensus QTLs were integrated into 72 pleiotropic unique QTLs. Two candidate genes, viz., GA signaling gene (BnRGA) and flowering gene (BnFT) located on chromosome A02, were identified as the most likely candidate genes associated with plant height regulation (Zhao et al. 2016).

Genome-wide association study for yield-related traits in *B. napus* also identified two candidate genes, a meristem initiation gene (*BnLOF2*) and a NAC domain transcriptional factor (*BnCUC3*) located on chromosome A07, which were identified to be associated with branch number (Zheng et al. 2017). Branch number is an important trait in plant architecture and found to be associated to crop yield. QTLs responsible for branch number were detected in a doubled haploid (DH) population and its reconstructed F_2 (RC-F_2) population (He et al. 2017). These findings provided

insights into the genetic basis for branching morphogenesis and may be valuable for optimizing architecture in rapeseed breeding. The combined study of GWAS and transcriptome sequencing on 21 haplotype blocks revealed the differential expression of 14 crucial candidate genes (such as Bna.MYB83, Bna.SPL5, and Bna. ROP3). Importance of these 14 candidate genes in developmental processes and biomass accumulation was demonstrated by functional annotation and expression pattern analysis (Lu et al. 2017).

Till date, only two silique-related trait genes have been cloned in rapeseed. One is the major QTL for seeds per silique, qSS.C9 (Zhang et al. 2012), which played a role in regulating the formation of functional female gametophytes (Li et al. 2015). Another is an auxin response factor 18 (ARF18) gene involved in both silique length and seed weight (SW) QTLs (Liu et al. 2015). ARF18 inhibited the activity of downstream auxin genes, which regulated silique wall development and determined SW via maternal regulation (Liu et al. 2015).

Progression of molecular and phenotypic diversification in resynthesized *Brassica juncea* (L) gene pool with determinate inflorescence has been reported by Kaur et al. 2014. In the determinate plants, apical meristems were transformed into pods. As the gene for determinacy has also been introgressed in *B. napus* and *B. carinata*, this had laid foundation for future breeding to aim at crop architectural modifications through determinacy in all three types of oilseed brassicas grown in the world (Kaur et al. 2014).

The Punjab Agricultural University (PAU), Ludhiana, India, has recommended the world's first determinate *B. carinata* oilseed variety PC-6 (Fig. 14.2) for general cultivation in Punjab under irrigated conditions. This medium tall variety, with oil content 40.1%, is resistant to pod shattering (species trait) and is suitable for mechanical harvest (Anonymous 2016).

Fig. 14.2 World's first determinate type *Brassica carinata* variety PC-6. (**a**) Synchronous maturity. (**b**) Suitable for mechanical harvest

14.3.5 MAS for Quality and Fatty Acid Modification

Enhancement in seed oil content is one of the important objectives in oilseed brassicas. About 1.0% increase in seed oil content is equivalent to an increase of 2.3–2.5% in seed yield in *B. napus* (Wang 2004). Rapeseed oil alone represents about 13.0–16.0% of the world vegetable oil production, and recent emphasis is being laid for producing feedstock oils for biodiesel too (Marjanović et al. 2016). Seed oil content is a typical quantitative trait, controlled by a large number of genes with additive effects and also under maternal and environmental influence (Banga and Banga 2009) and hence associated with inherent selection difficulties through conventional breeding procedures. To dissect the genetic basis of oil content and mapping of quantitative trait loci (QTLs) underlying this trait, will increase the efficiency and accuracy of conventional breeding programs for high oil content via marker-assisted selection (MAS). A larger population with significant differences in oil content gave a higher power detecting new QTLs for oil content, and the construction of the consensus map provided a new clue for comparing the QTLs detected in different populations (Wang et al. 2013). A large number of linkage maps were constructed in *B. napus* including some of which were used for QTL analysis of seed oil content (see Cheng et al. 2009 and references therein). However, most of the published linkage maps in rapeseed were based on molecular markers with little or no lipid-related gene information, which induced difficulties in discovering candidate genes for oil content by performing a direct genome comparison between B. *napus* and *A. thaliana* (Zhao et al. 2012). There have been a few reports on combining mapping of candidate genes related to seed oil content with genetic linkage maps constructed in QTL analysis to screen and identify candidate genes for seed oil content. A detailed RFLP-genomic map was used to study the genetics of oil, seed, and meal protein and the sum of oil and seed/meal protein contents in a recombinant doubled haploid population developed by crossing black- and yellow-seeded *Brassica juncea* lines (Mahmood et al. 2006). Tight linkage of three of these QTLs (SP-A1, SP-A9, SP-B4, O-A1, O-A9, O-B4), with opposite effects, pose challenge to the plant breeders for simultaneous improvement of negatively correlated ($r = -0.7^{**}$) oil and seed protein contents. However, one QTL for oil content (O-B3) and two for seed protein content (SP-B6, SP-C) were found to be unlinked, which offer the possibility for simultaneous improvement of these two traits. Epistatic interactions were significant for the quality traits, and their linkage reflected association among the traits. QTL *OilA1* for oil content in *Brassica napus* has been characterized and reported to be of particular interest for breeding purpose in China because 80% of Chinese cultivars do not carry this desirable allele (Chen et al. 2013a). Because of larger and complicated genome of *B. napus* than *A. thaliana*, in general, there is a lack of specific candidate genes for seed oil content directly derived from rapeseed. Moreover, it is time-consuming and laborious to finely map QTL for seed oil content. About 14 orthologous genes involved in lipid synthesis pathway in *A. thaliana* were mapped to 6 QTL regions in a *B. napus* DH population (Mahmood et al. 2006). These orthologous genes may be good candidate genes for seed oil content.

Beside oil content, quality of oil in terms of its fatty acid composition is very important. Rapeseed-mustard are high-erucic acid crops, containing >40% erucic acid in the oil. Due to the concerns over erucic acid content that stemmed from animal studies, high-erucic acid rapeseed oil used to be produced in North America solely in small quantities for industrial, nonfood use (Dupont et al. 1989). Trade disruptions during the Second World War made Canada examine its domestic edible oil resources, and rapeseed was chosen as a viable answer over other proven alternatives like sunflower or soybean, but there were concerns over a high proportion of erucic and eicosenoic acids which render the oil unfit for human consumption, and the meal was considered to inhibit growth in livestock and unsuitable for animal consumption. This led to continuous rapeseed research program with Saskatoon as the acknowledged birthplace of rapeseed research, and canola was developed from rapeseed through the use of traditional plant breeding techniques (Busch et al. 1994). In 1979, Canada registered the word "canola," and by definition, canola term specifies quality of oil with erucic acid (<2%) and glucosinolates (<30 umol/g defatted meal) (Mag 1983). In 1985, the US Food and Drug Administration granted canola oil "generally recognized as safe" (GRAS) status as a dietary component. The fatty acid composition of canola oil is consistent with its use as a substitute for saturated fatty acids (SFAs), in meeting the dietary goals recommended by many health associations: an average diet containing about 30% of calories as fat made up of less than 10% SFAs and 8–10% poly unsaturated fatty acids (PUFAs) in a ratio of linoleic to linolenic acids between 4:1 and 10:1, the remainder being monounsaturated fatty acids (MUFAs) (Molazem et al. 2013). No single oil meets these current recommendations for ratios of PUFA/monounsaturated/polyunsaturated fatty acid ratios as the sole source of cooking and salad oil (Dupont et al. 1989; Johnson et al. 2007; Gunston 2011). Presently, it is the most widely consumed food oil in Canada and has been approved for generally recognized as safe (GRAS) status by the Food and Drug Administration (FDA) of the United States Department of Health and Human Services. Sarkkinen et al. 1998 conducted a 6-month dietary intervention study in hypercholesterolemic adults and found that LDL-C levels were lowered (3.7%) from baseline in the rapeseed group; however, no significant differences were found in serum TC, LDL-C, HDL-C, and TAG levels between canola oil-based diets and diets with oils higher in SFAs. The positive effects of the high MUFA content of canola oil compared with SFAs on cardiovascular health through the regulation of plasma lipids and lipoproteins, susceptibility of low-density lipoprotein (LDL) oxidation, and insulin sensitivity have been well documented (Kris-Etherton et al. 1999 and Gillingham et al. 2011). As per studies of Gebauer et al. (2006), canola oil has been recommended for achieving daily n-3 FA requirements of 1 g/day for the treatment of existing cardiovascular disease. Concerns about the detrimental health effects of trans-fatty acids have led to a growing interest in using trans-free natural vegetable oils such as canola for deep-frying food too (Mozaffarian et al. 2006). Saini et al. (2016) developed and validated functional CAPS markers for the FAE genes in *Brassica juncea* for their use in marker-assisted selection. The paralogs of *FAE1 (fatty acid elongase 1)* gene were sequenced from low erucic acid genotype Pusa Mustard 30 and SNPs were identified through homologous alignment with

sequence downloaded from NCBI GenBank. Two SNPs in *FAE1.1* at position 591 and 1265 and one in *FAE1.2* at 237 were found polymorphic among low and high erucic acid genotypes (Saini et al. 2016). These markers for low erucic acid had also been utilized by *Brassica* team of Punjab Agricultural University (PAU), Ludhiana, India to expedite the canola breeding program. Cultivation of *B. napus*, in India is mainly confined to the states Punjab, Himachal Pradesh and Haryana due to climatic constraints. PAU. Ludhiana has developed canola-quality cultivars GSC 5 and GSC 6 of *B. napus*, and their release has precipitated significant interest among farmers due to higher price the canola produce fetches from millers as compared to conventional rapeseed-mustard genotypes (Kumar et al. 2009). Consistent efforts in canola breeding program further led to development of GSC-7 canola variety (Fig. 14.3) and released for commercial cultivation in Punjab in 2014 (Anonymous 2016). This variety is yielding higher than all commercial varieties of Punjab state and meets international standards for canola quality. This variety is an ideal blend of high yield and quality and created its niche in Punjab with more than 20,000 ha area under cultivation within span of 2–3 years after its release. The zero erucic mustard developed by Kirk and Oram (1981) has been utilized by Indian scientists for transferring zero erucic traits to Indian mustard varieties. Initial efforts concentrated on the development of genetic stocks for low erucic acid and low glucosinolate in the indigenous cultivars using exotic donor sources (Agnihotri et al. 2004). At PAU,

Fig. 14.3 High-yielding canola variety of *Brassica napus* variety GSC-7. (**a**) Flowering stage. (**b**) Pod-filling stage. (**c**) Seeds of GSC-7

Fig. 14.4 Canola-quality *Brassica juncea* variety RLC-3. (**a**) Flowering stage. (**b**) Pod-filling stage. (**c**) Yellow-colored seeds of RLC-3

Ludhiana, *B. juncea* lines with low erucic acid and high oleic acid have been developed (Banga et al. 1983) and led to the development of India's first *B. juncea* canola variety RLC-3 (Fig.14.4) having erucic acid of 0.5% in oil and 15 µmole/g defatted meal glucosinolate content and is yellow seeded. This variety has been recommended for general cultivation in Punjab state in year 2016 (Anonymous 2016).

B. napus has been extensively improved by breeders over the last five decades and hence canola quality traits *viz*., low seed erucic acid (>2%) and low glucosinolate content (>30 umoles/gm defatted meal) has been infused in almost all the modern rapeseed varieties (Friedt and Snowdon 2010). Owing to impressive health benefits of canola-quality oil, the improvement of seed quality traits for high oleic acid and low erucic acid and glucosinolate content remains the most important breeding goals in oilseed brassicas and, hence, mining of underlying QTLs, their fine mapping and map-based cloning of related QTLs has become domain of priority research in rapeseed-mustard.

Using F_1 microspore-derived DH population, Ecke et al. (1995) observed clear three-class segregation for erucic acid content, and the two erucic acid genes of rapeseed were mapped to two different linkage groups on the RFLP map. Two of the QTLs for oil content showed a close association in location to the two erucic acid genes, indicating a direct effect of the erucic acid genes on oil content. Relatively high levels of linolenic acid reduce the oxidative stability of the oil, and in order to identify

genes controlling the levels of erucic and linolenic acids, Thormann et al. (1996) identified two regions that accounted for nearly all of the phenotypic variation in erucic acid concentration and one region that accounted for 47% of the variation in linolenic acid concentration. The QTL associated with linolenic acid concentration mapped near a RFLP locus detected by a cDNA clone encoding an omega-3 desaturase, suggesting that the low linolenic acid content may be due to a mutation in this gene.

The unsaturated fatty acids of rapeseed oil, *viz*. oleic acid (C18:1), where 18 refers to the number of carbon atoms in the hydrocarbon chain and 1 refers to the number of double bonds between carbon atoms in that chain, linoleic acid (C18:2) and linolenic acid (C18:3) are synthesized as a result of the fatty acid desaturation pathway, in which double bonds are introduced, sequentially, starting from stearic acid (C18:0). In seed oil, these conversions are controlled by enzymes encoded by the genes *FAB2* (C18:0–C18:1), *FAD2* (C18:1–C18:2), and *FAD3* (C18:2–C18:3). After lowering of erucic acid in oilseed rape, modification of oleic acid and linolenic acid contents in seeds is one of the major goals for quality breeding. Smooker et al. (2011) reported the identification and mapping of candidate genes and QTL involved in the fatty acid desaturation pathway in *B. napus.* The analysis decoupled the effect of the segregation of the erucic acid-controlling loci and identify 34 QTL for fatty acid content of seed oil, 14 in the A genome and 20 in the C genome. The QTL indicated the presence of 13 loci with novel alleles inherited from the progenitors of the resynthesized *B. napus* that might be useful for modulating the content or extent of desaturation of polyunsaturated fatty acids, only one of which coincides with the anticipated position of a candidate gene, an orthologue of FAD2. Yang et al. (2012) identified all copies of FAD2 and FAD3 genes in the A and C genome of *B. napus* and its two diploid progenitor species, *B. rapa* and *B. oleracea*, through bioinformatic analysis and extensive molecular cloning.

Glucosinolates are sulfur-rich, anionic natural products that upon hydrolysis by endogenous thioglycosides (myrosinases) produce several different products (e.g., isothiocyanates, thiocyanates, and nitriles). The hydrolysis products have many different biological activities, e.g., as defense compounds and attractants (Halkier and Gershenzon 2006). As some glucosinolates may have detrimental effects on livestock palatability and health, great success was achieved by converting rapeseed into low erucic acid and low glucosinolate canola cultivars in the 1960–1970s, which paved a foundation for quality improved rapeseed to become one of the most three important oilseed crops in the world (http://apps.fas.usda.gov/psdonline/circulars/oilseeds.pdf). However, further decreasing glucosinolate content and modifying glucosinolate profiles in *B. napus* seed meal has been a long-term breeders' objective which has been hard to achieve using traditional breeding because of the complexity of the mechanisms involved in glucosinolate synthesis and regulation. Numerous markers associated with total GS content have been mapped and were identified on *B. napus* chromosomes N9, N12, and N19 (Uzunova et al. 1995; Howell et al. 2003; Zhao and Meng 2003). Recently, Liu et al. (2016) reported nine quantitative trait loci (QTLs) controlling total and major glucosinolate components localized on chromosomes A1, A8, A9, C2, C3, and C9 with the most abundant and significant QTLs residing on A9. It was worthwhile to use the co-segregating flanking markers of these QTLs for marker-assisted selection in practical canola breeding.

14.3.6 Genomic Approaches for Confering Disease Resistance

The production of oilseed brassicas is largely influenced by various biotic stresses which include fungal diseases (Oerke et al. 1994), bacterial diseases (Mathur and Swarup 1965), viral diseases (Hauser et al. 2000), nematodes (Jain 1978), and aphid pests (Razaq et al. 2011). Among these, fungal diseases are very important, and these infect oilseed brassicas right from seedling to fruit stage which leads to huge yield losses. Kolte (1985) reported that there are about 30 diseases which occur on these crops. On the basis of yield losses and wide distribution, *Alternaria* blight, white rust, sclerotinia stem rot, downy mildew, and powdery mildew are considered to be most important. The symptoms of *Alternaria* blight, sclerotinia stem rot, and white rust have been depicted in Fig.14.5. The development of resistant/tolerant varieties is the most economical and environment-friendly way to face these diseases. But the genotypic (host) variability is limited for all diseases except white

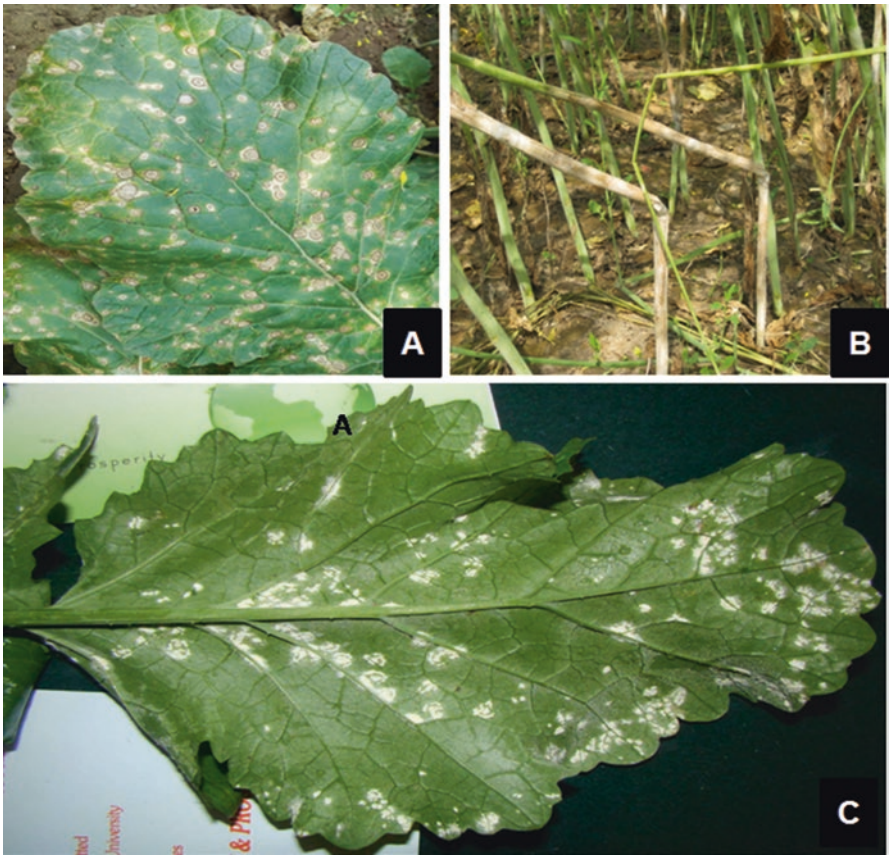

Fig. 14.5 Important diseases of oilseed brassicas. (**a**) Symptoms of *Alternaria* blight. (**b**) Symptoms of sclerotinia stem rot. (**c**) Symptoms of white rust

rust (Chauhan et al. 2011). Many qualitative and quantitative genes providing resistance to major diseases have been identified in brassicas. Qualitative disease resistance is often a total and conferred by single dominant R gene, whereas, quantitative disease resistance is governed by multiple genes having accumulative effects.

White rust is a widespread and destructive disease of *brassicas*. This disease is caused by *Albugo candida* (Pers.) Kuntze. Among oilseed brassicas, it is most important in mustard (*Brassica juncea* L. Czern. and Coss.) and turnip rape (*B. rapa* L.) (Saharan and Verma 1992; Kole et al. 1996). Liu (1996) reported yield losses of 30–60% in *B. rapa* due to white rust infection.

Due to obligate nature of the pathogen, use of exotic germplasm for resistance breeding has not been successful (Varshney et al. 2004). Therefore, marker-assisted selection would be helpful in the selection of resistant lines. Resistant genes against white rust have been identified and mapped in *B. junceae* (Cheung et al. 1998; Prabhu et al. 1998; Varshney et al. 2004; Massand et al. 2010), *B. napus* (Ferreira et al. 1995b; Somers et al. 2002), *B. rapa* (Kole et al. 1996), and *A. thaliana* (Borhan et al. 2001, 2008). Panjabi et al. (2010) have mapped two independent loci (AcB1-A4.1 and AcB1-A5.1) on linkage groups A4 and A5 in eastern European lines, Heera and Donskaya-IV, respectively, with PCR-based intron polymorphic (IP) markers. At PAU, Ludhiana, these markers are being used in MAS for transfer of white rust resistance gene.

Singh et al. (2015) validated the *Arabidopsis*-derived intron polymorphic (IP) markers At5g41560 and At2g36360, which were genotype non-specific gene markers and were closely linked with AcB1-A4.1 and AcB1-A5.1, respectively.

Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* is a major yield-limiting factor for oilseed brassicas worldwide and has wide host range. Due to the occurrence of virulent pathotypes, identification and breeding for host resistance have not been effective (Gea et al. 2012). Moreover, there were significant genotype-strain interactions (Garg et al. 2008). Therefore, QTL mapping can be helpful to identify loci providing resistance against sclerotinia stem rot. In *B. napus*, several of the resistance-related QTLs were found to be associated with A (A02, A03, A09) or C (C02, C04, C06, C07, C09) genomes of *B. napus* (Zhao et al. 2006; Yin et al. 2010; Wu et al. 2013). Wu et al. (2013) identified ten QTLs for stem resistance and three QTLs for leaf resistance against sclerotinia stem rot. They further identified major candidate gene BnaC.IGMT5 which is associated with major QTL SRC6 in *Brassica napus* by comparative mapping analysis with *Arabidopsis thaliana*. Wei et al. (2015) identified three loci associated with resistance in *B. napus* using genome-wide association studies. Gyawali et al. (2016) identified 21 loci conferring resistance against this disease using genome-wide association mapping in *B. napus*. As the suitable donor for resistance to this disease is not available within crop *Brassica*, Rana et al. (2017) developed introgression lines using wild species *B. fruticulosa* as donor parent in *B. juncea*. They detected ten significant marker trait associations, viz., CNU-m157–2, RA2G05, CNU-m353-3, CNU-m442-5, ACMP00454-2, ACMP00454-3, EIN2-3-1, M641-1, Na10D09-1, and Na10D11-1, in *B. juncea-B. fruticulosa* introgression lines (ILs). Molecular characterization of ILs using molecular cytogenetic studies and a set of transferable and candidate gene-based SSR markers is the first report.

In *B. oleracea*, Mei et al. (2013) reported separate QTLs for stem and leaf resistance against sclerotinia stem rot. Further, they identified that one major QTL for leaf resistance was present on C09 chromosome, whereas another QTL for stem resistance was at A09 chromosome. Molecular mapping technique can be used for deploying the resistance genomic segments from wild species which possess high levels of resistance against *S. sclerotiorum***.**

Another important disease of oilseed brassica is blackleg. The causal pathogen is *Leptosphaeria maculans*. Twelve of major race-specific R genes have been mapped on A genome of *B. napus* or *B. rapa* (*Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm7*, *Rlm9*, *LepR1*, *LepR2*, *LepR3*, *LepR4*) or the B genome of *B. juncea* (*LmJR1*, *LmJR2*) via linkage mapping, while two more have been assigned to B genome chromosomes of *B. juncea* and/or *B. nigra* (*Rlm6*, *Rlm10*) through marker-based assessment of recombinant or chromosome addition lines (reviewed in Neik et al. 2017). Several QTL responsible for quantitative resistance against *L. maculans* have been identified in *B. napus* (Jestin et al. 2011; Raman et al. 2012). Huang et al. (2016) detected 17 QTLs for adult plant resistance against *L. maculans* in *B. napus*. Six QTLs were found to be stable as these were less sensitive to environmental factors. Larkan et al. (2015) identified and delineated four significant and stable QTL suitable for MAS of quantitative blackleg resistance in *B. napus*. A cluster of cysteine-rich receptor-like kinase genes was associated with a significant field-derived QTL on chromosome A01.

Clubroot disease is caused by *Plasmodiophora brassicae*, an obligate biotroph chytrid. This disease caused yield loss up to 50% in Canada (Strelkov and Tewari 2005) and 20–30% in China (Chai et al. 2014). Two resistant genes (*Crr1a* and *CRa*) have been cloned in *B. rapa* against clubroot disease. Hatakeyama et al. (2013) cloned and characterized *Crr1a* gene in *B. rap*, which confers resistance to clubroot. The second gene, *CRa*, was cloned by Ueno et al. (2012). It is a candidate gene encoding a TIR-NBS-LRR protein. Pyramiding of these genes into high-yielding cultivar will increase the durability of resistance against this disease. Major R genes and QTLs for major diseases in oilseed brassica have been reviewed by Neik et al. (2017). The breeding for disease resistance using marker-assisted selection will be helpful and accelerate the breeding program. The genomic regions identified from the wide species for resistance against various diseases will be valuable sources for future breeding program by using introgression lines.

14.3.7 QTLs for Nutrient Uptake

Efficient mineral nutrient utilization by crop plants is important in modern day agriculture. Minerals are essential for various cellular activities in plants. Plant accumulates minerals in aboveground organs and is a complex trait (Clemens 2001). Mobilization of nutrients from soil, uptake by roots, translocation and redistribution within plant, and import and deposition in the organs are major processes for mineral accumulation in plant. To develop nutrient-efficient crop varieties, plant breeder has to understand the genetic and physiological basis of mineral concentration.

The improvement of mineral content by plant breeding, also referred to as biofortification (Bouis 2002), requires knowledge about the genetic variation for this trait and genetic markers linked to relevant genes (reviewed in Ghandilyan et al. 2006). Under stressful nutrition conditions, plant growth, development, and yield will be altered due to change in availability, uptake, and utilization of nutrient elements (Gupta 2005). The individual chromosomal regions controlling complex traits can be identified using quantitative trait locus (QTL) mapping (Alonso-Blanco and Koornneef 2000). Among various minerals, phosphorus is a limiting factor globally in crop production. The plant has to change root morphology and architecture under low P conditions. QTLs associated with leaf and seed, P and phytate concentration, and primary root growth responses to low P availability had been reported among various *Brassicaceae* species (Bentsink et al. 2003; Zhao et al. 2007, 2008; Hammond et al. 2009; Yang et al. 2010; Ding et al. 2012). Detection of these loci will help to breed *Brassicas* with improved phosphorus efficiency.

14.4 Genome Approaches for Accelerated Breeding in Oilseed Brassicas

Reports for significant heterosis for some agronomic characters such as plant height, leaf area, and yield in *B. napus* (Lefort-Buson et al. 1987) and the possibility of homogeneous production (for mechanical harvesting) in *B. oleracea* have stimulated interest in the development of hybrid cultivars (Renard et al. 1992). Up to 200% hybrid vigor of yield in *B. napus* and *B. juncea* had been reported (Tingdong et al. 1990, Jain et al. 1994). But to exploit the advantages of heterosis on a commercial scale, an effective pollination control system to produce F_1 hybrid seeds is essential. In diploid species (*B. oleracea and B. campestris*), hybrid cultivars were mostly produced by using self-incompatibility genes. But the genetic background effects, requirement of bud pollination and generally incomplete incompatibility impede commercial hybrid seed production. Numerous controls are therefore necessary to check about the good genetic quality of seeds. This leads to high seed costs to the growers. In allotetraploid species (*B. napus and B. juncea*), hybrid seed production is impeded by the preferential autogamous character of these crops. Thus, an effective system of cross-pollination is necessary for hybrid rapeseed breeding (Renard et al. 1992). The male sterility induction in brassica can be helpful by which plants are not able to produce functional pollen. Genes from *Bacillus amyloliquefaciens* have been transferred into Indian mustard *B. juncea* to induce male sterility using genetic transformation method (Jagannath et al. 2002). Genetic transformed varieties are not accepted by many in the world, and, hence, development of male sterile systems through molecular approaches could be an alternative and an efficient option. In India, mostly cytoplasmic male sterility (CMS) system is being used to develop hybrids. Oilseed rape hybrid, PGSH 51, based on *tournefortii* system, was the first India-bred oilseed brassica hybrid, released on commercial scale in 1994

(Banga et al. 1995). Later, many hybrids have been released by public and private sector and have shown 15–20% yield advantage across locations over commercial varieties.

Comparisons between *Brassica* and *Arabidopsis* have identified significant genomic regions of synteny and duplication. Comparative mapping led to clone *Rfo*, a single radish nuclear gene, which suppressed Ogura (*ogu*) cytoplasmic male sterility (Brown et al. 2003). S45A, a double recessive mutant at both the *BnMs1* and *BnMs2* loci in *Brassica napus*, found to produce no pollen in mature anthers and no seeds by self-fertilization. The development of the pollen was seriously affected, resulting in complete male sterility. The map-based cloning of the *BnCYP704B1–1* and *BnCYP704B1–2* genes is a successful example of cloning gene using *Arabidopsis* data in *B. napus* (Yi et al. 2010). A dominant point mutation in a RINGv E3 ubiquitin ligase homologous gene leads to cleistogamy in *Brassica napus* (Yun-Hai et al. 2012). The researchers reported cloning and characterization of the Bn-CLG1A (CLG for cleistogamy) gene and the Bn-clg1A-1D mutant allele responsible for the cleistogamy phenotype. Cleistogamy trait could be useful in developing genetically modified (GM) cultivars with low risk of gene flow to non-GM varieties (Daniell 2002).

Compared to the traditional breeding methods, doubled haploid production can save years off the breeding process. Haploid breeding has become widely adopted for oilseed brassica breeding. Most of the *Brassica napus* cultivars in Canada had been derived from DH breeding (Ferrie and Mallers 2011). Isolated microspore culture techniques have been reported to produce DH plants in *Brassica* (Palmer et al. 1996), but there have been few reports of this technique being applied to *B. juncea*. With the discovery by Ravi and Chan (2010) on selective modification in centromere histone gene, *CENH3* in *Arabidopsis* has provided the molecular mechanism underlying selective loss of chromosomes and has opened new opportunity for constructing haploid inducer (HI) lines through genetic engineering. A novel method of haploid induction through genetic engineering of the CENH3 has been later demonstrated in *Arabidopsis* and Banana. Recently, Watts et al. (2017) explored the possibility of CENH3-mediated genome elimination in B. *juncea*, a close relative of *Arabidopsis*, using RNAi-mediated gene silencing. But, RNAi approach to suppress *CENH3* gene to develop haploid inducer lines yielded a very low frequency of haploids (0.16% in maize reported by Kelliher et al. 2016 and 0.65% in *Brassica juncea* by Watts et al. 2017). However, the results by Watts et al. (2017) supported the principle of tailswaping of *CENH3* leading to haploid induction in mustard. Knockout mutants of *CENH3* impose a major limitation in polyploidy crops like *B. juncea* where more than one copy of *CENH3* is expected. Fu et al. 2018 provided a new insight for generation of homozygous lines in vivo using a single-step approach through the creation of a new *Brassica* artificial hybrid, a *Brassica* allooctaploid (AAAACCCC, $2n = 8\times = 76$), by interspecific crossing and genome doubling. Crosses between *B. napus* as female and *Brassica* allooctaploid as pollen donor yielded maternal doubled haploid of *B. napus*, which were identified based on phenotype, ploidy, and molecular analysis.

14.5 Conclusion

The generation of genomics resources and translational research in the last decade extended great support for *Brassica* crop improvement. The genome sequencing of the model plant *Arabidopsis thaliana* and later *Brassica rapa* and *B. oleracea* has provided the basis for the genomic and comparative studies among the *Brassica* species. Genome collinearity has great potential to facilitate the identification of candidate genes in *Arabidopsis* homologous to the genes controlling traits of economic importance in Brassicas. Comparative mapping of *Brassica* species with the *Arabidopsis* genome also helps in understanding conserved genetic architecture and genome evolution and functional analysis of genes for important agronomic traits in Brassicas. The cost-effective and efficient methods for single nucleotide polymorphism (SNP) discovery by using next-generation sequencing (NGS) techniques have further strengthened the genomics research. Identification of genomic regions from wild species conferring resistance to various diseases and cloning of gene(s) supported the accelerated breeding approach for biotic stresses. Gene expression profiling through transcriptome analysis has laid strong foundation for *Brassica* improvement program specially breeding for biotic and abiotic stresses.

Effective pollination control systems for hybrid development, resistance to pod shattering in canola, coupling of determinacy traits for mechanical operations, and genomic-assisted breeding for higher productivity, quality, and tolerance to biotic and abiotic stresses are major upcoming research areas to keep pace with climate challenges.

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