Satbir Singh Gosal
Shabir Hussain Wani *Editors*

Accelerated Plant Breeding, Volume 1

Cereal Crops

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Editors

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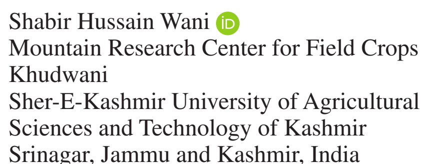
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This book is dedicated to Dr. Gurdev Singh Khush—An acknowledged hero of rice revolution.

Dr. Gurdev Singh Khush

Dr. G.S. Khush was born in a small village Rurkee of district Jalandhar, Punjab, India. He obtained B.Sc. degree in 1955 from 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 IR 64), 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 World Food Prize, which is regarded as equivalent to the Nobel Prize in agriculture. Other international awards received by him include; Japan Prize, Rank Prize From UK, Wolf Prize in Agriculturefrom Israel, Golden Sickle Award from Thailand, and Mahathir Science Award from Malaysia. He has been bestowed Honorary Doctorate degrees by 16 Universities, including Punjab Agricultural University (India), Cambridge University (UK), Ohio State University (USA), McGill University (Canada). Dr. Khush has been elected to world's most prestigious academies such as 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, 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. Dr. Khush 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, 24 invited review articles and published over 250 research papers in referred journals. Through a generous donation, Dr. Khush has established 'Dr. Gurdev Singh Khush Foundation for Advancement of Agricultural Sciences' at Punjab Agricultural University, Ludhiana-141004, India.

Foreword

The plant breeding started with the process of "selection" which led to the domestication of about 500 plant species through centuries as a maiden act of artificial selection of plants for human consumption. Such a form of plant breeding started simply as an art because little was known about the scientific basis of the plant traits. Over the years, plant breeding was put on sound scientific basis all the way through contributions from Mendelian genetics. Plant breeding which was earlier considered "art and science for changing and improving the characteristics of plants" is now greatly reliant on science. Plant breeding methods such as selection, hybridization, polyploidy, and induced mutations lead into superior crop varieties with high yield, quality, and resistance to biotic/abiotic stresses. The world food grain production, which was 918 million tons during 1961, has been enhanced to 3076 million tons during 2017. Similarly, the world population which was 3070 million in 1961 increased to 7600 million in 2017. During this period, the food grain production increase (235.08%) was more as compared to population increase (147.56%). Thus, the per capita food grain production (2.99 qtls. in 1961) has been enhanced to 4.05 qtls. in 2017. The development and release of high yielding crop varieties and hybrids has played a major role in increasing food grain production world over. In the current scenario of changing climate and WTO, farmers desire for new varieties, suitable not only for local consumption but also for export purposes. The pursuit for sustainable agriculture can benefit greatly from powerful new technologies that accelerate the process of plant breeding. *Accelerated Plant Breeding: Cereal Crops*, edited by Drs. Satbir Singh Gosal and Shabir Hussain Wani, is aimed to describe the innovative methods which supplement/complement the conventional breeding methods for precision and accelerated crop improvement. The book includes chapters prepared by specialists and subject experts on different cereal crops/aspects in relation to accelerated breeding. The first chapter introduces various methods/technologies currently being used to accelerate the breeding process for early release of varieties. Further specific chapters dealing with data management and advanced quantitative genetics technologies have been included. Recent "speed breeding" and "genomic selection" methods have been described in separate chapters. Besides, chapters dealing with accelerated breeding of major cereal crops such as rice, bread

and durum wheat, maize, barley, finger millet, barnyard millet, and sorghum have been included. Thus, this volume provides state-of-the-art information on new innovative methods/technologies which hold significant promise to speed up the process of plant breeding. I feel that this book will be very useful for students, research scholars, plant breeders, and scientists working in the areas of genomics, cellular/molecular biology, and biotechnology. I congratulate the editors of this book Dr. S.S. Gosal and Dr. Shabir Hussain Wani for getting valuable contributions from the selected experts working on important cereal crops.

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Preface

Plant breeding is a continuous process which results into new crop varieties with improved characteristics. In the current scenario of climate change and global warming, there is rapid emergence of new races of insect-pests and new pathotypes of disease-causing agents. Minor insect-pests/pathogens are rapidly emerging as major ones. Heat and drought stresses are becoming serious threats. Under current WTO regime, farmers desire new high yielding varieties not only for local consumption but also for export purposes. Thus, plant breeders have to constantly develop new stainable varieties with high yield, quality, resistance to diseases and insect-pests, and tolerance to abiotic stresses. Now, the cultivator also demands for high yielding varieties possessing durable and multiple disease resistance, early maturity, higher harvest index, dual purpose forages, varieties with nutrient-use efficiency/water-use efficiency, wider adaptability, suitable for mechanized harvesting, with better shelf life, better processing quality, with improved minerals, vitamins, amino acids, proteins, antioxidants, and bioactive compounds. Using conventional approaches, in a self-pollinated crop like wheat, it takes about 10–12 years for the development and release of the new variety. Therefore, an efficient improvement in the existing cultivars is necessary to meet the challenge and rising food demand. The quest for sustainable agriculture can benefit greatly from powerful new technologies that accelerate plant breeding. In the current era of Breeding 4.0, where specific parts in the genome can be targeted, technological advances along with the data revolution greatly improve the capacity of plant geneticists and breeders to develop durable varieties. Therefore, this book will be highlighting the innovative techniques/technologies such as doubled haploidy, micropropagation, somaclonal variation, embryo culture, marker-assisted selection, marker-assisted background selection, genomic selection, high-throughput genotyping, high-throughput phenotyping, reverse breeding, transgenic breeding, shuttle breeding, speed breeding, genome editing, advanced quantitative genetics technologies, and intentional and standardized data management presently being used to supplement/complement the conventional approaches for accelerating plant breeding. This volume provides an authoritative review account of various innovative methods/approaches and improvement in important cereal crops that has been made in the recent past. The

book includes chapters prepared by specialists and subject experts on different crops/aspects in relation to accelerated breeding. The first chapter introduces various methods/technologies currently being used to accelerate the breeding process for early release of varieties. An imperative chapter dealing with data management and advancements in data analytics has been especially included. Likewise, advanced quantitative genetics technologies for accelerating plant breeding have been included in separate chapter. “Speed breeding” and “genomic selection” methods have been described in separate chapters. Two chapters exclusively deal with rapid improvement in rice. Likewise, separate chapters cover different approaches for accelerating breeding in bread and durum wheat. Two chapters deal with maize; the first covers doubled haploid technology and second deals with bio-fortification. Some other cereals such as barley, finger millet, and barnyard millet have been dealt in separate chapters. A chapter on dual purpose cereal, sorghum, has also been included.

The book provides state-of-the-art information on new innovative methods/technologies to speed up plant breeding. We earnestly feel that this book will be highly useful for students, research scholars, and scientists working in the area of plant breeding, genomics, cellular/molecular biology, and biotechnology at universities, research institutes, and 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 who 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 Nature Switzerland AG, Gewerbestrasse 11, 6330 Cham, Switzerland, for their careful and speedy publication of this book.

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

Contents

1 Accelerated Breeding of Plants: Methods and Applications 1
Satbir Singh Gosal, Dharminder Pathak, Shabir Hussain Wani, Suruchi Vij, and Mamta Pathak

2 Speed Breeding: Methods and Applications 31
R. Abdul Fiyaz, B. C. Ajay, K. T. Ramya, J. Aravind Kumar, R. M. Sundaram, and L. V. Subba Rao

3 Genomic Selection in Cereal Crops: Methods and Applications 51
Mohammed Saba Rahim, Abhishek Bhandawat, Nitika Rana, Himanshu Sharma, Afsana Parveen, Prashant Kumar, Akansha Madhawan, Ashita Bisht, Humira Sonah, Tilak Raj Sharma, and Joy Roy

4 Data-Driven Decisions for Accelerated Plant Breeding 89
Saritha V. Kuriakose, Ravindra Pushker, and Ebenezer M. Hyde

5 Advanced Quantitative Genetics Technologies for Accelerating Plant Breeding 121
Dharminder Bhatia

6 Haploid Production Technology: Fasten Wheat Breeding to Meet Future Food Security 139
Masahiro Kishii and Sukhwinder Singh

7 Recent Advances in Chromosome Elimination-Mediated Doubled Haploidy Breeding: Focus on Speed Breeding in Bread and Durum Wheats 167
H. K. Chaudhary, A. Badiyal, N. S. Jamwal, P. Sharma, N. V. Manoj, and K. Singh

8 Acceleration of the Breeding Program for Winter Wheat 191
Alison M. R. Ferrie and Patricia L. Polowick

9 Genomics, Biotechnology and Plant Breeding for the Improvement of Rice Production 217
Kshirod K. Jena and Sung-Ryul Kim

10 High-Frequency Androgenic Green Plant Regeneration in *Indica* Rice for Accelerated Breeding 233
Sanghamitra Samantaray, Byomkesh Dash, Sudhansu Sekhar Bhuyan, Parmeswaran Chidambaranathan, Jawahar Lal Katara, Ram Lakhan Verma, B. N. Devanna, Krishnendu Chattopadhyay, Awadesh Kumar, and A. Anandan

11 Doubled Haploid Technology for Rapid and Efficient Maize Breeding. 257
Vijay Chaikam and B. M. Prasanna

12 Biofortification of Maize Using Accelerated Breeding Tools. 293
R. K. Khulbe, Arunava Pattanayak, and Devender Sharma

13 Efficient Barley Breeding 309
Satish Kumar, Madhu Patial, and Rajiv Sharma

14 Finger Millet (*Eleusine coracana* (L.) Gaertn.) Genetics and Breeding for Rapid Genetic Gains 365
S. Ramesh and T. V. Krishna

15 Breeding Advancements in Barnyard Millet 391
Salej Sood, Dinesh C. Joshi, and A. Pattanayak

16 Sorghum Improvement Through Efficient Breeding Technologies. 411
D. Balakrishna, Avinash Singode, B. Venkatesh Bhat, and Vilas A. Tonapi

Index. 437

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Satbir Singh Gosal has been Bursary holder, The Royal Society, London, is a former Director at the School of Agricultural Biotechnology and Ex-Director of Research at Punjab Agricultural University, Ludhiana, India. He was an Honorary Member of the Board of Assessors (Australian Research Council, Canberra), Biotechnology Career Fellow, The Rockefeller Foundation, USA, and President of the Punjab Academy of Sciences. He has published more than 200 research papers in refereed journals and 35 book chapters. He has coauthored one textbook and coedited five books including three with Springer.

Shabir Hussain Wani received his PhD in Genetics and Plant Breeding from Punjab Agricultural University. He has published over 100 peer-reviewed papers and edited 13 books on plant stress physiology, including seven with Springer. He also served as a Review Editor for Frontiers in Plant Science from 2015 to 2018. He is currently an Assistant Professor at the Mountain Research Centre for Field Crops of the Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir in India.

Chapter 1
Accelerated Breeding of Plants: Methods and Applications

Satbir Singh Gosal, Dharminder Pathak, Shabir Hussain Wani, Suruchi Vij, and Mamta Pathak

1.1 Introduction

Agriculture is facing enormous challenges of food and nutritional security, climate volatility and natural resource depletion. The quest for sustainable agriculture can benefit greatly from powerful new technologies that accelerate plant breeding for rapid release of superior crop varieties. Plant breeding that is used for improving genetic performance of crops, earlier considered "art and science for changing and improving the characteristics of plants", is now heavily dependent on science component. Plant breeding is a continuous process which results in new varieties with improved yield and quality of the produce besides enhancement of tolerance to insect pests and diseases. In the current scenario of climate change and global warming, there is rapid emergence of new races of insect pests and new pathotypes of disease-causing agents. Minor insect pests/pathogens are rapidly emerging as major ones. Heat and drought stresses are becoming serious threats. Under current WTO regime, farmers wish for new high-yielding varieties suitable not only for local consumption but also for export purposes. Plant improvement has been largely focused on improving yield, quality, resistance to diseases and insect pests and

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tolerance to abiotic stresses. Nowadays, farmers demand for high-yielding varieties of different crops possessing durable and multiple resistance, early maturity, higher harvest index, dual-purpose forages, varieties with nutrient-use efficiency/water-use efficiency, wider adaptability and suitability for mechanized harvesting, with better shelf life, better processing quality and improved minerals, vitamins, amino acids, proteins, antioxidants and bioactive compounds. Conventional breeding approaches at this juncture seem inadequate to meet the growing demand for superior varieties. For instance, in a self-pollinating crop like wheat, from crossing of elite parental lines, 4–6 generations of selfing are required to fix the traits for the subsequent agronomic evaluation of the new lines. Thus it takes 10–12 years to develop and release a new variety. During the past 10 years, significant advances have been made, and accelerated methods have been developed for precision breeding and early release of crop varieties. Innovative techniques such as doubled haploidy, micropropagation, somaclonal variation, embryo culture, marker-assisted selection, marker-assisted background selection, genetic mapping, genomic selection, high-throughput genotyping, high-throughput phenotyping, reverse breeding, transgenic breeding, shuttle breeding, speed breeding, advanced quantitative genetics technologies and genome editing are now increasingly being used to supplement/complement the conventional approaches for efficient improvement in the existing cultivars.

1.2 Doubled Haploidy

In self-pollinated crops, an exceedingly long period is required to bring together desirable gene combinations from different sources in homozygous form. Generally, it takes 8–10 years to develop stable, homozygous and ready-to-use materials from a new cross. Whereas, in cross-pollinated crops, due to inbreeding, it becomes difficult to develop vigorous inbreds (true-breeding lines) for hybrid seed production programmes. In this regard, haploids possessing gametic chromosome number are very useful for producing instant homozygous true-breeding lines. In conventional breeding, the early segregating generations involve variation attributable to both additive and non-additive genetic effects (Khush and Virk 2002), whereas doubled haploid (DH) lines exhibit variation only of additive genetic nature, including additive x additive type of epistasis, which can be easily fixed through a single cycle of selection. The elimination of dominance effects leads to high narrow-sense heritability and availability of sufficient seed of each DH line, which allows for replicated testing. Thus, in contrast to relatively large segregating populations in conventional genetic studies, less DH lines are required for the purpose of selection of preferred recombinants. For instance, in rice, about 150 DH lines derived from F_1, instead of 4000–5000 F_2 plants, are sufficient for selecting desirable genotypes. Production of haploids/doubled haploids through anther culture from F_1 rice plants results in true-breeding plants in less than 1 year, which otherwise takes seven to eight generations through conventional methods (Fig. 1.1). Haploids can be induced in vivo and in vitro. In maize (*Zea mays* L.), haploids are commonly produced by in vivo haploid induction system (inducer lines), and the method has been commercially

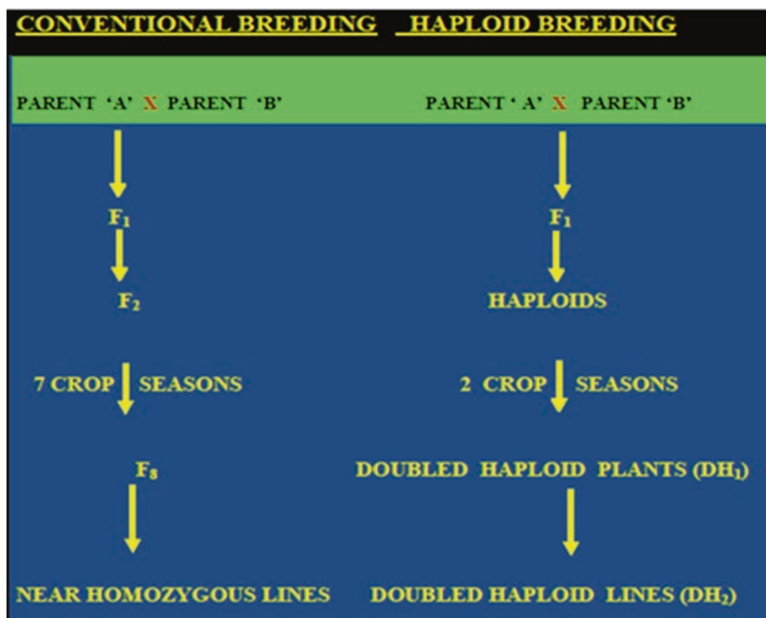

Fig. 1.1 The period required to develop homozygous lines through conventional and haploid breeding

exploited by both public and private sector organizations. DH populations are ideal for genetic mapping and allow the development of high-density marker maps that can then be exploited in quantitative trait loci identification. Genetic mapping using DHs has been used for locating genes controlling traits such as yield, quality, agronomy and abiotic and biotic stresses. DHs now feature in cultivar production in a number of crops, and breeding time is considerably reduced. Hence, worldwide use of DH technology as an accelerated approach to crop improvement has become routine by many breeding companies and laboratories leading to the development of almost 300 new varieties (Sood and Dwivedi 2015).

1.2.1 Methods of Haploid Production

The important methods for production of haploids include:

1. Anther culture
2. Isolated microspore culture
3. Unpollinated ovary culture
4. Embryo rescue from wide crosses
5. Use of haploid inducer lines
6. Gene engineering for induction of haploids

1.2.1.1 Anther Culture

Anthers are cultured in liquid or on semisolid agar medium (Gill et al. 2003) where they may directly give rise to embryoids or may lead to callus formation before differentiation. The embryoids develop into haploid plantlets or doubled haploids in some crops (because of spontaneous doubling of chromosomes during callus proliferation). Haploids may be treated with colchicine to obtain fertile, doubled haploid homozygous plants for field testing and selection. The technique of anther culture was first developed in *Datura* by Guha and Maheshwari (1964, 1966). Flower buds at an appropriate stage are collected from healthy greenhouse or field-grown plants. The collected flower buds are usually wrapped in plastic bags and kept in refrigerator at 4 °C for 7–10 days for cold treatment. Flower buds are surface sterilized with 0.1% $HgCl_2$ for 8–10 min, and anthers are carefully dissected out of the flower buds and inoculated on the medium. Cultures are incubated at 25 ± 1 °C under diffused light conditions. In general, the cultured anthers exhibit callusing after 2–6 weeks. About a month-old calli are made to regenerate into plants. Anther culture systems have been developed for several important cereal crop plants such as wheat (De Buyser et al. 1986; Laurie and Reymondie 1991; García-llamas et al. 2004; Mujeeb-Kazi et al. 2006), maize (Obert and Barnabas 2004; Ambrus et al. 2006; Bernardo 2009) and rice (Raina and Zapata 1997; Senadhira et al. 2002; Sarao et al. 2003; Grewal et al. 2006; Zhahg-Yi et al. 2008; Mishra and Rao 2016; Tripathy et al. 2019). Hundreds of cultivars are either in test or have been released in rice, wheat and maize. Further, the doubled haploid approach is increasingly being used for rapid development of populations for QTL mapping and construction of genetic linkage maps for traits of interest.

1.2.1.2 Isolated Microspore Culture

Microspore culture (Nitsch and Nitsch 1969; Ferrie and Caswell 2011; Shariatpanahi and Ahmadi 2016) has several advantages over anther culture because microspores are haploid single cells that can readily be genetically manipulated. Unlike anther culture, microspore culture eliminates the participation of diploid tissues (anther wall and connective tissues). The two methods of pollen isolation are (a) naturally shed pollen in the culture medium after pre-culture of anthers and (b) mechanical means by crushing or magnetic stirring. The naturally shed pollen is known to result in more calli and plantlets than mechanically isolated pollen of rice and barley. In case of rice, large microspores (50–58 μm) with thin pink-coloured outer walls produced embryos, whereas the division of small (40 μm) microspores with thick cell walls was not observed (Cho and Zapata 1990). Addition of glutamine, proline at 1 mM concentration and ficoll 10% (w/v) into the culture medium has shown beneficial effects during the isolated microspore culture in the liquid medium. Microspore/pollen culture has been used in *Hordeum vulgare* (Hoekstra et al. 1993), wheat (Hu and Kasha 1997; Scagliusi 2014) and oats (Sidhu and Davies 2009).

1.2.1.3 Ovary Culture

Ovary culture is an alternative approach to develop haploids where anther/pollen culture fails to produce haploids (Chen et al. 2011; Rakha et al. 2012). In contrast to anther culture, ovary culture is less effective because there is only a few embryo sacs per ovary as compared to thousands of microspores/pollen per anther. Using this technique, haploids are induced from megaspores, through gynogenesis as in wheat (Getahun et al. 2013). It is well established in maize where haploids are developed by culturing unpollinated ovaries (Tang et al. 2006). Further, the method has also been extended to other crops, such as barley and rice. Genotypic differences have been observed for the development of gynogenic calli. In rice, *japonica* types have been found to be more responsive than genotypes of *indica* types. Like anther culture, the success of ovary culture is largely dependent on the developmental stage of the ovary. Success has been reported with ovaries ranging from uninucleate to mature embryo sac stages. Use of growth regulators to promote gynogenesis and to inhibit the proliferation of somatic tissues has been very critical for ovary culture (Zhou and Yang 1981). Rice ovaries failed to enlarge in the absence of MCPA (2-methyl-4-chlorophenoxyacetic acid). An increase in MCPA concentration from 0.125 to 8 mg/L favoured ovary swelling. However, the rate of induced embryo sacs has generally been higher than for microspores, and the frequency of green plant regeneration has also been higher than from anther cultures. But ovary culture has been successful only in a few species. Ovary culture has also been successful in watermelon (Zou et al. 2018).

1.2.1.4 Embryo Rescue from Wide Crosses

Embryo rescuing from wide crosses in some crops serves as an alternative route to haploidy. Moreover, the system is less prone to gametoclonal variation owing to the absence of redifferentiated callus phase. The phenomenon is based on the elimination of full set of chromosomes of one of the parents during the embryo development in vitro.

Bulbosum Method

This method was first developed for production of haploids in diploid barley by Kasha and Kao (1970). The haploids are produced from inter-specific crosses between *Hordeum vulgare* (female) and *H. bulbosum* (male). Zygote induction is fairly high, and the chromosomes of *H. bulbosum* are rapidly eliminated from the developing embryos. Developing endosperm also aborts after about 2–5 days of growth which necessitates the rescuing of embryos in order to complete their development. Embryo culture using nutritionally rich medium results in complete haploid plants of *H. vulgare*, and chromosome doubling is induced in the established plants. Barclay (1975) extended this method to wheat where haploids of wheat were

obtained through embryo culture from *Triticum aestivum* cv. Chinese Spring x *H. bulbosum* cross. However, this method is restricted to wheat varieties possessing "kr" crossability genes which are responsible for the elimination of *H. bulbosum* chromosomes. Further, it has been concluded that the loss of CENH3 from *H. bulbosum* preceded uniparental chromosome elimination during the development of *H. vulgare* × *H. bulbosum* hybrid embryos (Sanei et al. 2011). This discovery of CENH3, in chromosome elimination, suggests the possibility of producing haploids through CENH3 modification.

Haploid Production in Wheat from Wheat × Maize and Wheat × *Imperata cylindrica* Crosses

Complete wheat haploid plants using wheat x maize system by employing in vitro culture of wheat spikelets, 2 days after pollination, were obtained (Laurie and Reymondie 1991). This method has been successfully extended to durum wheat (O'Donoughue and Bennett 1994) and to field-grown bread wheat by daily injecting 2,4-D (125 ppm) into pollinated tillers for 3 days followed by embryo culturing 15 days after pollination with maize (Bains et al. 1995; Verma et al. 1999; Ding et al. 2019). Haploids produced through any of these methods have been used to produce doubled haploid lines through colchicine treatment. The doubled haploids produced from F_1 plants represent a set of new recombinant lines in homozygous state which can be used for commercial cultivation or for further breeding purposes. Likewise, another system, wheat x *Imperata cylindrica*—a new chromosome elimination-mediated system—has also been developed for production of wheat haploids using cogon grass (*Imperata cylindrica*) as pollen parent (Chaudhary et al. 2002, 2005, 2013; Mahato and Chaudhary 2015). The doubled haploid (DH) system has been widely used (Patial et al. 2019) in wheat improvement, and several varieties of DH origin have been released.

1.2.1.5 In Vivo Haploid Production Using Inducer Lines

In maize, the Stock-6 has been generally used to produce haploids for inbred line production. The original Stock-6 inducer line was created with haploid induction (HI) rates of 2.52% and 1–2% when Stock-6 was self-fertilized or outcrossed as a male, respectively (Coe 1959). Selective breeding has improved the HI potential of Stock-6 derivatives to 7–15% (Eder and Chalyk 2002; Xu et al. 2013). In maize, development of new haploid inducers with high haploid induction rates (HIRs) and adapted to different target environments have facilitated increased adoption of DH technology in new environments. Haploid identification is being optimized using different genetic markers and non-genetic methods (including automation), thereby reducing the cost and time spent in haploid identification (Kermicle 1969; Weber 2014).

1.2.1.6 Gene Engineering for Induction of Haploids

It is an emerging aspect involving gene editing using CRISPR and other new technologies. During the year 2010, it was reported that haploid *Arabidopsis thaliana* plants were obtained through the introduction of the single genetic alternation in the centromere histone H3 (CEN H3). Further it was suggested that haploids can be induced either through genetic engineering of CEN H3 N-terminal tail or histone-fold domain or by replacing CEN H3 with an ortholog (Ravi and Chan 2010; Wang et al. 2019b). Now a wide variety of point mutations in CENH3, inducible by chemical agents, have been shown to result in haploid induction on crossing with wild-type CENH3 plants. These CENH3-variant plants grow normally, are fully fertile on self-pollination and may be present in existing mutagenized collections (Britt and Kuppu 2016). Likewise, mutation of a pollen-specific phospholipase gene, MATRILINEAL (MTL), has been shown to trigger the haploid induction (HI) in maize, which presents another promising HI approach by the editing of MTL in plants. Haploid inducer-mediated genome editing (IMGE) approach has been developed (Wang et al. 2019a) that utilizes a maize haploid inducer line carrying a CRISPR/Cas9 cassette targeting for a preferred agronomic trait to pollinate an elite maize inbred line and to generate genome-edited haploids in the elite maize backgrounds. Homozygous pure doubled haploid lines with the preferred trait improvement could be generated within two generations, thus bypassing the lengthy procedure of recurring crossing and backcrossing used in conventional breeding for integrating a desirable trait into elite commercial backgrounds.

1.3 Micropropagation

Micropropagation of plants is now one of the best and most successful examples of the commercial application of tissue culture technology. Propagation of plants from very small plant parts (0.2–10 mm) under in vitro conditions in the laboratories/polyhouses is called micropropagation. It has three major advantages: (1) It ensures true-to-type plants, i.e. identical to mother plant (cloning); (2) it results in rapid and mass multiplication (about 1–10 times/cycle of 2 weeks each) (Fig. 1.2) of elite clones/varieties which are otherwise difficult to multiply using conventional methods; and (3) it is independent of seasonal constraints. In vegetatively propagated crops such as sugarcane and potato, during hybridization after making crosses, the F_1 seeds are space planted to get F_1 hybrid plants. Such plants are propagated vegetatively to grow next generations for agronomic evaluation. But due to low rates of multiplications, it takes longer to develop sufficient planting material for replicated and multi-location evaluation. In this regard, micropropagation helps in quick bulking of the plants for evaluation and early release of varieties (Gosal et al. 1998; Gill et al. 2006; Sood et al. 2006). Further, it also helps in quick bulking of mutants, haploids, triploids and independent transgenic events for their early evaluation.

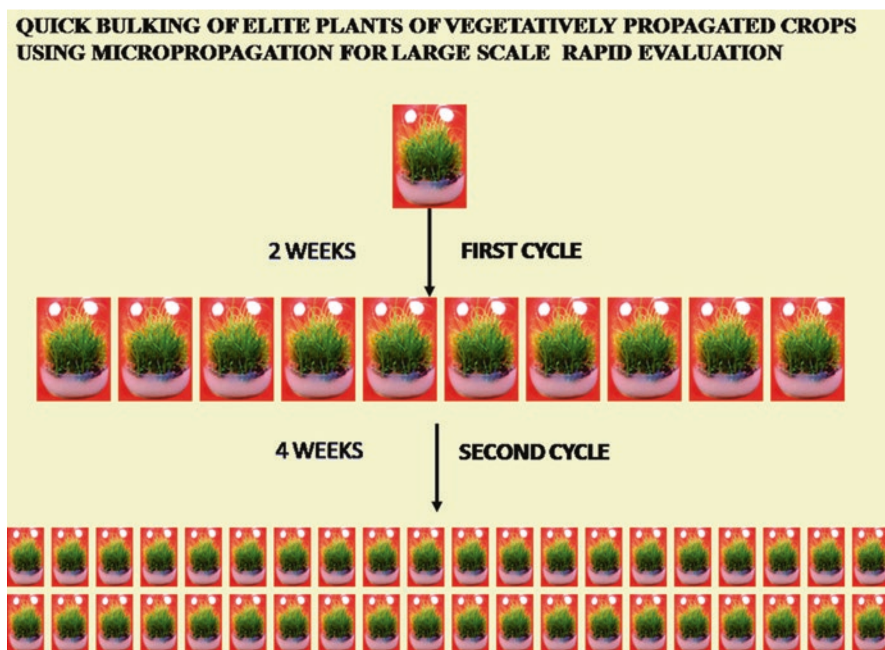

Fig. 1.2 Quick bulking of elite plants of vegetatively propagated crop using micropropagation through tissue culture for large-scale rapid evaluation

1.4 Somaclonal Variation

Variation among plants derived from the in vitro somatic cell cultures is called somaclonal variation. It may be genetic or may result from culture-induced epigenetic changes (Larkin and Scowcroft 1981). The epigenetic changes are expressed at cell culture stage, but these usually disappear when plants are regenerated or reproduced sexually. It, therefore, provides a novel mechanism to generate new genetic variation for plant improvement. Using this technique, many million cells (potential plants) can be evaluated in a single Petri dish, which is virtually difficult, if not impossible, to be adopted at whole plant level in the field. Moreover, in vitro selection also reduces the chances of diplontic selection. Somaclonal variants can be identified through field evaluation for characters like yield that cannot be evaluated at single-cell level or through cell selection that involves application of suitable selection pressure like that of some toxins to permit preferential survival of variant cells. This simple and cost-effective technique possesses a huge potential for the improvement especially of vegetatively propagated plant species and, of course, seed-propagated crop plants with narrow genetic base. Several interesting and potentially useful traits have been recovered using this method in maize (Zheng et al. 2004), rice (Araujo and Prabhu 2004; Cristo et al. 2006; Elanchezhian and Mandal 2007; Mandal et al. 2016), wheat (Ahmed and Abdelkareem 2005; Sabry

et al. 2005; Akhtar et al. 2015; Abu-Gammie et al. 2016) and millet (Mhatre et al. 2016). Recovery of novel variants that either do not exist or are rare in the natural gene pool, for example, atrazine resistance in maize, improved lysine and methionine contents in cereals. Genetic, cytogenetic and molecular evidences for increased recombination frequency through cell culture have now been provided (Larkin et al. 1993). Tissue culturing of wide hybrids also helps in breaking undesirable linkages and achieving introgression from alien sources. Several new varieties have been developed through somaclonal variation in many crops. However, under numerous situations, low plant regeneration ability and the lack of correspondence in expression of the trait in field-grown plants are the major problems.

1.5 Embryo Culture

Embryo culture helps in overcoming problems associated with seed germination and seed dormancy. During generation advance, grain filling period takes several weeks to produce viable mature seeds. For instance, in bread wheat, seed-to-seed period in Punjab, India, is about 160 days, out of which about 25–30 days are required for the seed maturity after fertilization (about 16% of life cycle duration). Likewise, in early maturing *indica* rice varieties, the seed-to-seed period in the Indian Punjab is about 125 days, out of which about 25–30 days (about 20% of life cycle duration) are required for the seed maturity after fertilization. Thus, culturing immature embryos and raising plants help in saving time in generation advance (Dağüstü et al. 2012). Further combining embryo culture with greenhouse facility, more than one generation can be advanced in 1 year. During distant hybridization (crosses involving wild species or parents from different genera) when parents are genetically diverse, the developing endosperm degeneration leads to embryo abortion and failure of the cross. Immature embryos are aseptically excised and cultured in vitro using suitable culture medium and optimum cultural conditions. The plantlets thus obtained are transferred to soil in the greenhouse. It has been a practical approach (Gosal and Bajaj 1983; Sharma et al. 1996) to develop inter-specific and inter-generic hybrids in wheat (Kaur et al. 2002).

1.6 Transgenic Breeding

In fact, transgenesis has emerged as an additional tool to carry out "single-gene breeding" or "transgenic breeding" of crops. Unlike conventional hybridization, only the cloned gene(s) controlling useful traits are being introduced without cotransfer of unwanted genes from the donor. The recipient genotype is least disturbed, which eliminates the need for frequent backcrosses. Above all, the transformation method provides access to a large gene pool, as the useful gene(s) may come from viruses, bacteria, fungi, insects, animals, human beings and

unrelated plants and even from chemical synthesis in the laboratory. Among various gene transfer methods, *Agrobacterium* and "particle gun" are being commonly and widely used. Now attempts are being made to develop transgenic varieties resistant to insect pests, diseases, herbicides and abiotic stresses, such as drought, low and high temperature, salts and heavy metals, and also to develop transgenic varieties possessing better nutrient-use efficiency and better keeping nutritional and processing qualities (Gosal and Wani 2018; Araus et al. 2019).

A useful gene such as *Cry* 1Ac (Bt) is introduced in a tissue culture-responsive genotype using appropriate gene transfer method. The regenerated T_0 plants are subjected to molecular (PCR, real-time PCR, Southern, Western) analyses to check the presence, copy number and expression of the introduced gene(s). The elite events are identified and multiplied for further evaluation under contained conditions. Stable transgenic lines are then subjected to biosafety and large-scale field evaluation and finally released as transgenic variety if found superior to check variety. For developing more transgenic varieties carrying the same trait, there is no need to undergo the process of genetic transformation again and again. The popular transgenic variety is crossed as donor parent with another popular (non-transgenic) variety as recipient genotype, and stable lines with useful transgene(s) are developed using conventional backcross breeding. Thus, backcrossing process is completed in order to give an existing line, known as the recurrent parent, the transgene(s) from the donor parent (Fehr 1987). The backcrossing process is repeated as needed to recover the phenotype of the recurrent parent while minimizing the chromatin contribution from the donor, as only the transgene(s) are of interest. Integrating doubled haploidy/molecular markers with transgenic breeding further accelerates the development of new transgenic varieties/hybrids.

1.7 Speed Breeding

Speed breeding, also known as accelerated breeding, is an additional tool available to speed up plant breeding. The concept was first tested by NASA, along with Utah State University, in an attempt to evaluate food production under the constant light on space stations, starting with wheat. A group of scientists, led by University of Queensland, Australia, expanded it to several crop plants, alongside the investigation of additional growth parameters including the harvest of immature seed. Accordingly, speed breeding greatly shortens generation time and accelerates crop breeding process. Now speed breeding can be used to achieve up to 6 generations per year for spring wheat (*Triticum aestivum*), durum wheat (*T. durum*), barley (*Hordeum vulgare*), chickpea (*Cicer arietinum*) and pea (*Pisum sativum*) and 4 generations for canola (*Brassica napus*), instead of 2–3 under normal glasshouse conditions (Ghosh et al. 2018; Watson et al. 2018). Speed breeding in fully enclosed, controlled environment growth chambers can accelerate plant development for research purposes, including phenotyping of adult plant traits, mutant studies and

transformation. The use of additional lighting in a greenhouse environment allows rapid generation cycling through single seed descent (SSD) and potential for adaptation to larger-scale crop improvement programmes. Cost saving through light-emitting diode (LED) supplemental lighting is also outlined. Speed breeding methods can be extended to other crops in conjunction with other modern crop breeding technologies, including high-throughput genotyping, genome editing and genomic selection to accelerate the rate of crop improvement. Speed breeding protocols could be applied to shorten breeding cycles and accelerate research activities also in orphan crops (Chiurugwi et al. 2019).

1.8 Shuttle Breeding

Shuttle breeding uses varied ecological environments to develop improved varieties with better adaptability. Alternate generations of breeding materials are grown under different environmental conditions. Shuttle breeding approach is being used, more particularly, by CIMMYT and IRRI for the improvement of wheat, maize and rice. Breeding for rust resistance in wheat was a slow process taking up to 10 or 12 years. Dr Norman Borlaug recommended a new method of shuttle breeding to speed up the development and take advantage of both of Mexico's growing seasons. He sought to grow wheat in the cooler central highlands near Mexico City in the summer and then shuttle selected plants to the warmer northwestern Yaqui Valley during the winter for a second round of breeding and selection (Caroline 2014). The different latitudes, elevations and climates of these two locations allowed Borlaug and his colleagues to breed and select plants twice in 1 year. Later, shuttle breeding has been the main approach of the CIMMYT, which involves breeding at two contrasting locations in Mexico, wide adaptation, durable rust and septoria resistances, international multi-site testing and appropriate use of genetic variation to enhance yield gains of subsequently produced lines (Ortiz et al. 2007). However, at the beginning of the twenty-first century, this "cultivar assembly line" approach needs fine-tuning to address crop needs under increasingly adopted resource-conserving practices, as well as those related to nutritional requirements of the end users. International wheat improvement will therefore focus on the targeting of traits in respective mega-environments and the use of participatory methods, especially in marginal environments. The main features of this wheat improvement strategy include the introduction of new and novel sources of genetic variation through wild species, landraces and, potentially, the use of transgenes for intractable traits. This variation will be combined using international shuttle breeding, and increased breeding efficiency will be achieved through marker-aided methods, more targeted use of crop physiology, plant genetics, biostatistics and bioinformatics. Likewise, Tanio et al. (2006) has reported rapid generation advancement on heading traits of Japanese wheat.

1.9 Genomic Selection

It is a type of marker-assisted selection in which genetic markers covering the whole genome are used so that all quantitative trait loci (QTL) are in linkage disequilibrium with at least one marker (Goddard and Hayes 2007). This approach has become feasible with the availability of a large number of single nucleotide polymorphisms (SNPs) revealed by genome sequencing and innovative methods to efficiently genotype large number of SNP. Methods, models and perspective of genomic selection in plant breeding have been thoroughly discussed by several researchers (Lorenz et al. 2011; Crossa et al. 2017; Wang et al. 2018; Robertsen et al. 2019). Genomic selection (GS) has promised to overcome the limitations of MAS for quantitative traits. The objective of GS is to determine the genetic potential of an individual instead of identifying the specific QTL. Genomic selection makes use of the combined approaches of advanced traditional breeding, development of double haploid (DH) lines, concept of field design and quantitative population genetics integration. GS selections with molecular tool pushed more genetic gain particularly over MAS for background and foreground selection, identification of variant alleles and multiple cycle selection by crossing (Bassi et al. 2016). The major advantage of this technology is reduced number of genetic markers obtained at population generation advancement of field study. Flanking genes with single marker analysis by genome-wide high-throughput marker is more efficient than genome-wide background selection by high-throughput marker (Bassi et al. 2016). It is possible that sometimes due to lack of marker availability and nature of trait, it has not lead to genetic gain.

CIMMYT maize breeding research in sub-Saharan Africa, India and Mexico has shown that genomic selection can reduce the breeding interval cycle to at least half the conventional time and produces lines that, in hybrid combinations, significantly increase grain yield performance over that of commercial checks. Using marker-assisted selection, it has been difficult to improve the multigenic characters such as abiotic stresses and grain yield. Whereas, the genomic selection approach has paved the way to overcome these limitations.

1.10 Reverse Breeding

Reverse breeding (RB) is another plant breeding technique for directly producing parental lines from heterozygous plant. RB creates complementing homozygous parental lines through engineered meiosis. The method is based on reducing genetic recombination in the selected heterozygote by eliminating meiotic crossing over. Male or female spores produced by such plants contain combinations of non-recombinant parental chromosomes which can be cultured in vitro to produce homozygous doubled haploid plants (Dirks et al. 2009; Erikkson and Schienmann 2016). Complementary parents can be selected from the doubled haploid and used

to reconstitute the heterozygote. In the case of plant species where an extensive collection of breeding lines is still lacking, RB can certainly accelerate the development of improved varieties. Reverse breeding in conjunction with high-throughput genotyping can go a long way for accelerating crop breeding as high-throughput genotyping speeds up the process of identification of complementing parents in populations of DHs in early stages. Another reverse genetics method was developed to identify and isolate deletion mutants for targeted plant genes. Deletion mutant libraries were generated using fast neutron bombardment. DNA samples extracted from the deletion libraries were used to screen for deletion mutants by polymerase chain reaction (PCR) using specific primers (Li et al. 2001). Other major advantage of reverse breeding is that it makes possible the selection of superior hybrids. Large populations of plants can be generated and screened, and likely plants can be regenerated indefinitely without prior knowledge of their genetic constitution (Kumari et al. 2018). So far, more work has been done on *Arabidopsis*, and there is a need to focus on field crop plants to harness the benefits of this technology.

1.11 Genome Editing

Genome editing or genome engineering or gene editing is a fast-emerging technology that gives scientists the ability to change an organism's DNA. Genome editing is a type of genetic engineering in which DNA is inserted, deleted, modified or replaced in the genome of a living organism. Unlike earlier gene transfer methods that randomly insert genetic material into a host genome, genome editing targets the insertions to site-specific locations. Plant breeding is entering a new era with the emergence of various sequence-specific nucleases (SSNs). The SSNs mediate efficient editing of genomes of various mammalian and plant species. These SSNs induce double-stranded breaks at specific chromosomal sites followed by their repair through non-homologous end-joining pathway resulting in nucleotide insertions and deletions. If homologous donor templates are available at the site of double-stranded breaks, homology-directed repair (HDR) can also occur (Symington and Gautier 2011). The different types of SSNs include zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR/Cas system. Among these, CRISPR/Cas system based on a bacterial CRISPR-associated protein-9 nuclease (Cas9) from *Streptococcus pyogenes* has generated great enthusiasm (Cong et al. 2013; Grohmann et al. 2019) which is now most commonly and widely used for genome editing in plants. To cleave the target DNA, Cas9 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 include Cas9 enzyme (CRISPR-associated endonuclease), SpCas9, dCas9, SaCas9 and Cas9D10A. Introduction of CRISPR/Cas construct into plant cells is carried out through various direct (vectorless) and indirect (vector-mediated) gene transfer methods. Whereas, *Agrobacterium*

tumefaciens is commonly used in plants. After introduction of CRISPR/Cas construct into the genome of target plant, the resulting mutations are identified using methods such as mismatch cleavage assays, nuclease assay, PCR-RE assay (Beumer et al. 2008), quantitative PCR high-resolution melting (qPCR-HRM) curve analysis technique (Yu et al. 2014a) and sequencing. Successful genome editing has been carried out in different crops including wheat (Zhang et al. 2016, 2017; Gil-Humanes et al. 2017; Liang et al. 2017) and rice (Zhang et al. 2014; Huang et al. 2018; Miao et al. 2018; Mishra et al. 2018). All these reports show that superior plant backgrounds can be directly improved by editing of the target genes without hybridization with the donor parents.

1.12 Marker-Assisted Selection

In varietal development programme, plant breeder selects the plants from the segregating generations based on their phenotypes. However, phenotype of a plant does not always reflect its genotype. This is especially true for the quantitative traits including yield which have complex inheritance and are substantially affected by environmental factors. This results in the reduced selection efficiency. Moreover, many of the traits like nematode resistance, tolerance to various abiotic stresses, etc. are sometimes unreliable, time-consuming, costly and difficult to screen. Here, marker-assisted selection (MAS) comes to the aid of plant breeder. MAS is a type of indirect selection, where selection is based on the genotype of the plant and not on its phenotype. This has become possible due to the development of DNA-based markers. Molecular marker(s) linked to desired trait are identified following the standard procedure of gene/QTL tagging. Now, the marker(s) linked to gene/QTL of interest and not the trait per se is monitored during the segregating/backcross generations. Thus, MAS obviates the need for phenotypic screening and relies solely on the detection (presence/absence) of the associated marker(s), thus overcoming the limitations of phenotypic selection/screening. There are five major applications of MAS in plant breeding: marker-assisted evaluation of breeding material, marker-assisted backcrossing, marker-assisted pyramiding, marker-assisted early generation selection and combined marker-assisted selection (Collard and Mackill 2008). The first successful use of MAS in plant breeding was demonstrated for soybean cyst nematode (*Heterodera glycines*) resistance in soybean (Concibido et al. 1996). Since then, a large number of varieties have been developed and commercialized worldwide in various crops such as wheat (Unnat PBW343 in India, Biointa 2004 in Argentina, Lilian in Canada); rice (Cadet and Jacinto in the USA; Angke and Conde in Indonesia; Swarna-Sub1, Samba Mahsuri-Sub1, Improved Samba Mahsuri, Improved Pusa Basmati 1, Improved Pusa RH10, PB3 and PR127 in India); maize (Vivek QPM 9 in India); barley (Sloop SA and Sloop Vic in Australia); soybean (Sheyenne in the USA); bajra (HHB 67-2 in India); and white bean (Verano in Puerto Rico).

1.13 Marker-Assisted Background Selection

Simply inherited traits are being transferred to the elite genotypes following backcross breeding since the last almost 100 years. The ultimate objective of such breeding schemes is to fully recover the genotype of recurrent parent along with the incorporated gene(s)/trait(s) of interest from the donor parent. The general procedure involves the creation of F_1 hybrid between the recipient and the donor parents, development of backcross generations following five or six backcrosses with the recurrent parent and few rounds of self-fertilization for the fixation of the introduced gene(s). The target trait is selected in each BCF_1 or BCF_2 generation depending on dominant or recessive nature of the allele under transfer. The entire process of backcross procedure takes at least nine crop seasons or more if the recessive gene is to be transferred. The situation becomes more complex in terms of linkage drag if the donor parent is an unadapted/wild species. Another limitation of this scheme is that, by the time the genotype of the elite recurrent parent is recovered, other advance lines derived from the ongoing breeding programmes may outperform the improved version of the old crop variety (recurrent parent).

The retrieval of recipient parent genotype may be greatly enhanced through marker-assisted background selection (MABS) strategy. The MABS involves detection of the target gene/QTL in the BC generations/progenies through the identification of one or more tightly linked molecular markers (foreground selection). This is followed by background selection with the aim to identify individual plants with maximum recovery of the recurrent parent genotype for further backcrossing or selfing. The distance between the linked marker and the gene/QTL should be <5 cM for efficient foreground selection. The ideal situation would be when the marker is a part of the gene itself, thus ensuring 100% linkage with the gene/QTL. Other factors affecting the MABS efficiency include number of individuals in the BC generations, number of polymorphic markers employed, genome coverage, etc. Using MABS strategy, the recurrent parent genotype may be retrieved in just two or three backcrosses as compared to five or six backcrosses conducted in the conventional breeding programmes. Marker-assisted background selection has been successfully used in rice resulting in the development and release of improved versions of landmark varieties, e.g. IR64-Sub 1, Swarna-Sub 1, Improved Samba Mahsuri, etc.

1.14 Genetic Mapping

Chromosomal theory of inheritance revealed parallelism of chromosomal behaviour during meiosis and the Mendelian factors, thus giving an indication about the presence of genes onto the chromosomes. However, Thomas Hunt Morgan (1911) provided unequivocal experimental evidence about the location of white-eye gene on X chromosome of *Drosophila* and set the stage for the construction of genetic linkage maps. A little later, his student Alfred Sturtevant (1913) reported the world's first

genetic linkage map in *Drosophila*. Determination of relative position of genes on the chromosome and the distance between them is referred to as genetic mapping. Genetic maps are based on the recombination frequency between the genes, which is a function of the distance between them. Such maps do not reveal the physical distance between the genes in terms of base pairs. For this, one has to construct the physical maps, and sequencing the entire genome is the ultimate physical map of an organism. All the classical linkage maps were based on the phenotypic characters. Biochemical markers such as isozymes too were used in the genetic analyses. Morphological and protein-based markers have their own limitations. The breakthrough came in 1980 with the invention of the first DNA-based marker, i.e. restriction fragment length polymorphism (RFLP), by Botstein and co-workers (Botstein et al. 1980). Another revolution was witnessed in the mid-1980s with the availability of polymerase chain reaction (PCR) (Mullis et al. 1986). PCR-based markers like random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), single nucleotide polymorphism (SNP), etc. were extensively used for the construction of molecular linkage maps. The availability of whole genome sequence of a large number of crop plants has led to the construction of SNP arrays which have been used for the development of high-density molecular linkage maps and mapping of genes/QTLs (Hulse-Kemp et al. 2015; Li et al. 2016; Cai et al. 2017; Liu et al. 2018; Kumar et al. 2019; Ramesh et al. 2019; Song et al. 2019). The major advantages of gene mapping include precise tracking of the gene/QTL in the segregating generations, marker-aided selection and map-based gene cloning. Various steps for gene mapping include development of a suitable mapping population, documentation of parental polymorphism using appropriate molecular markers, phenotyping of the mapping population and finally establishing the link between gene/QTL and the marker(s). F_2, $F_{2:3}$, recombinant inbred lines (RILs), backcross inbred lines (BILs), near isogenic lines (NILs), doubled haploids (DHs), nested association mapping (NAM) population, multi-parent advanced generation intercross (MAGIC) lines, chromosome segment substitution lines (CSSLs), etc. may be used as mapping populations.

1.15 Single Seed Descent Method

Single seed descent (SSD) breeding scheme of advancing segregating generations of a cross was initially proposed by Goulden (1939) and, later on, modified by Brim (1966). In this method, no selection is practised in the first segregating generation, i.e. F_2. Single seed from each of the F_2 plants is randomly taken, bulked and advanced to F_3 generation. Similarly, in the F_3 generation, single seed from each of the F_3 plant is randomly taken, and the process is repeated in the later segregating generations (F_4, F_5, F_6). In the pedigree method which is the most common and popular breeding scheme followed across the crops, selection is recommended and practised in the main season only. However, in the case of SSD methodology, multiple crop seasons can be taken making use of off-season nursery, greenhouse facilities, etc. as

no selection is involved. Further, the process can also be accelerated through the use of embryo rescue technique, where the immature embryo is cultured to obtain the next generation, hence obviating the need to obtain mature seeds in vivo. For example, up to eight and nine generations have been obtained in wheat (Zheng et al. 2013; Yao et al. 2017) and barley (Zheng et al. 2013) using embryo culture. The end product of SSD methodology is the faster development of homozygous lines which have undergone several rounds of recombination.

1.16 High-Throughput Phenotyping

Most of the traits of economic importance especially yield are quantitative in nature. These are under complex genetic control and are greatly influenced by environment. A high degree of accuracy in recording phenotypic data is the key for the precise identification of the QTLs and measurements of their effects, hence establishing phenotype-genotype relationships correctly. Advances in DNA marker assays and sequencing technologies have facilitated the generation of large amount of genotypic data at reasonable cost. However, manual recording of comprehensive phenotypic data for large number of traits especially at different crop stages/time intervals under field conditions poses a challenge in terms of accuracy, cost and time. The matching developments in high-throughput phenotyping technologies have been sluggish, though the last two decades have witnessed substantial progress in the improvement of these technologies. Based on the extant technologies, plant phenotyping may be conducted using spectral reflectance/absorbance, thermography, optical imaging and platforms/methodologies for root system analysis (Walter et al. 2015). Here, we give a brief account of the use of various high-throughput phenotyping technologies for the measurement of different plant traits. Using automated image capture and analysis device, Dias and co-workers (2011) recorded seed germination and pre-emergence growth at extreme temperatures in model plant *Medicago truncatula* and identified the QTLs for these traits. Seed protein, oil, carbohydrate, fatty acid and amino acid contents were estimated in thousands of soybean mutant lines employing near-infrared (NIR) spectroscopy (Bolon et al. 2011). Optical cameras mounted on unmanned aerial vehicle (UAV) is a potential technique for monitoring crop growth/architecture. MK-Oktokopter, a UAV system, mounted with high-resolution red-green-blue (RGB) camera has been used in barley to capture multi-temporal stereo images which were processed to obtain plant height values (Bendig et al. 2013). Nuclear magnetic resonance (NMR) relaxometry has been employed to detect variations during the process of senescence, and relationship between cell hydration and leaf senescence with NMR signal was established in *Brassica napus* (Musse et al. 2013). Employing non-destructive, rapid and cost-effective hyperspectral reflectance approach, accurate assessment of some leaf photosynthetic and biochemical traits (specific leaf area, [CO_2]-saturated rate of photosynthesis, leaf chlorophyll, nitrogen and sucrose content), which are vital indicators of stress response, has been demonstrated in a diverse set of maize

germplasm (Yendrek et al. 2017). Canopy temperature—an important indicator of water status— affects many physiological parameters and ultimately crop yield. A reliable and inexpensive method of measuring canopy temperature using airborne thermography with a radiometrically calibrated thermal camera for measuring canopy temperature in large field experiments has been developed and demonstrated in wheat (Deery et al. 2016). Conventional methods of studying root traits are very cumbersome. Novel non-destructive and more accurate techniques for better insight into root architecture, growth and responses to different environmental factors are available (Fang et al. 2009; Clark et al. 2011). Impedance flow cytometry, a reliable, efficient and non-destructive technique, has successfully been used for the analysis of pollen quality (Heidmann et al. 2016). A recent comprehensive review describing high-throughput phenotyping (HTP) of plant traits, various HTP platforms, software packages, etc. is available (Pratap et al. 2019).

1.17 High-Throughput Genotyping

Genetic analysis has evolved from looking at the phenotypes to deciphering the genetic information at the whole genome level. Tremendous progress has been made in sequencing technologies since 1977 when the initial reports by Sanger et al. (1977) and Maxam and Gilbert (1977) were published. Of the various DNA-based markers, single nucleotide polymorphisms (SNPs) are the most informative and widely used markers for genotyping. Detection of SNPs using high-throughput sequencing technologies is termed as genotyping by sequencing (GBS). GBS is simple, specific, highly reproducible and rapid owing to simultaneous detection of SNPs and genotyping (Chung et al. 2017). The GBS is based on reduced representation sequencing (RRS) and whole genome resequencing (WGR) methods. The RRS-based methods include restriction site-associated DNA sequencing (RADseq), Elshire genotyping by sequencing (Elshire GBS), two-enzyme GBS, double-digest RAD sequencing (ddRAD), sequence-based genotyping (SBG), ezRAD, restriction fragment sequencing (RESTseq), specific length amplified fragment sequencing (SLAF-seq), 2bRAD, multiplexed shotgun genotyping (MSG), reduced representation library (RRL), complexity reduction of polymorphic sequences (CRoPSTM) and RAD capture (Rapture), whereas WGR-based methods are sliding window WGR, parental inference WGR, parental inference WGR with individualized model and skim genotyping by sequencing (Scheben et al. 2017 and references therein). GBS methods have been widely used in crop plants for the construction of high-density molecular linkage maps [Chutimanitsakun et al. 2011 (barley), Poland et al. 2012b (barley and wheat), Ward et al. 2013 (red raspberry), Zhou et al. 2014 (peanut), Davik et al. 2015 (cultivated strawberry), Guajardo et al. 2015 (sweet cherry), Scaglione et al. 2015 (kiwifruit), Moumouni et al. 2015 (pearl millet), Crawford et al. 2016 (prairie cordgrass)]; QTL mapping [Zou et al. 2012 (sorghum); Yang et al. 2013, 2015 (lupin); Spindel et al. 2013, 2015 (rice); Bastien et al. 2014 (soybean); Lin et al. 2015 (wheat); Balsalobre et al. 2017 (sugarcane)]; genomic

selection in wheat (Poland et al. 2012a, Rutkoski et al. 2014); genome-wide association studies (GWAS) [Huang et al. 2012 (rice), Crossa et al. 2013 (maize), Li et al. 2013 (maize), Hwang et al. 2014 (soybean)]; diversity and phylogeny analyses [(Bus et al. 2012 (rapeseed), Escudero et al. 2014 (*Carex* species), Fu et al. 2014 (yellow mustard)], etc.

Availability of genome sequences has led to the development of SNP chips in many crops such as wheat (Cavanagh et al. 2013; Wang et al. 2014; Winfield et al. 2016; Allen et al. 2017), rice (Chen et al. 2014; Yu et al. 2014b; Singh et al. 2015; McCouch et al. 2016), maize (Ganal et al. 2011; Unterseer et al. 2014; Rousselle et al. 2015), cotton (Hulse-Kemp et al. 2015; Cai et al. 2017), soybean (Song et al. 2013; Lee et al. 2015), barley (Comadran et al. 2012), *Brassica* (Clarke et al. 2016), chickpea (Roorkiwal et al. 2017), oat (Tinker et al. 2014), cowpea (Close et al. 2015), peanut (Pandey et al. 2017), potato (Hamilton et al. 2011; Vos et al. 2015), rye (Bauer et al. 2017), ryegrass (Blackmore et al. 2015), sunflower (Livaja et al. 2016), tomato (Sim et al. 2012), etc. These SNP chips have been used for development of molecular linkage maps (Hulse-Kemp et al. 2015), QTL mapping (Li et al. 2016; Raja et al. 2017; Zhu et al. 2018; Kumar et al. 2019; Ramesh et al. 2019; Song et al. 2019), diversity analysis (Zhao et al. 2010; Hinze et al. 2017), GWAS (Zhao et al. 2011), etc.

1.18 Future Prospects

Innovative techniques such as doubled haploidy, micropropagation, somaclonal variation, embryo culture, marker-assisted selection, marker-assisted background selection, genetic mapping, genomic selection, high-throughput genotyping, high-throughput phenotyping, reverse breeding, transgenic breeding, shuttle breeding, speed breeding, single seed descent method**,** advanced quantitative genetics technologies and genome editing, in conjunction with the conventional plant breeding methods, can be exploited to accelerate plant breeding for early release of crop varieties.

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Chapter 2
Speed Breeding: Methods and Applications

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2.1 Introduction

Crop yields need to increase in the future in order to meet the demand of increasing human population. It was predicted that human population is expected to grow by 25% over the next 30 years and would reach around ten billion (Hickey et al. 2019). The development of newly improved cultivars with higher yield and resistance to major pests and diseases is needed quickly to meet the increasing demand. Although several conventional methods are available for increasing the yield, the current pace of genetic gain or increase in productivity is insufficient to meet the demand. Globally, crop breeding programmes have become stagnant and localized and operate under the influence of environment. The plant breeders are under tremendous pressure to improve breeding programmes considering time as a unit of function to enhance crop yields with better disease and pest resistance and resilience to climate change. Climate change is considered to be the most important threat to world food security in the future as it is unpredictable, and it leads to increase in CO_2 levels, temperature, outbreaks of pest and diseases, minor pests that became major problems, frequent floods, droughts, reduction in global crop yields and deterioration of quality of the agricultural produce. Furthermore, these extreme weather conditions add burden on world food security and pressure on plant scientists to transform the breeding programme to cope up with the extreme conditions (Peng et al. 2004; Nelson et al. 2009; Wassmann et al. 2009; Newton et al. 2011; Lobell and Gourdji 2012; IPCC 2014; Sreenivasulu et al. 2015; Zhao et al. 2017; Van Oort and Zwart 2018).

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Present-day crop improvement programmes are endowed with several innovative technologies like automated high-throughput phenotyping technologies which enable screening of large populations in shorter times with accuracy and high-throughput, automated genotyping methodologies. With the advent of high-throughput genotyping platforms, plant scientists can utilize genomic information for aiding in the selection process, making easier the processes of trait dissection, fine mapping and gene discovery, and in development of improved crop varieties (Ramya et al. 2013; Bassi et al. 2016; Araus et al. 2018). Conventional breeding programmes around the world have led to release of many improved cultivars or varieties in many crops over the last five to six decades. Nevertheless, the progress is very slow as the process involves crossing the parental lines, followed by 4–6 generations of selfing in order to fix the lines for evaluation of agro-morphological and yield traits. This is time-consuming for crops that are often limited to only 1–2 generations per year (Hickey et al. 2019). To enhance the productivity level of various crops from the present level, it is therefore imperative to shorten the generation time and hasten the breeding programme. In order to accelerate the breeding process in crop plants, based on experiments by NASA, USA, to grow plants in space, researchers in the University of Queensland developed a new concept called 'speed breeding', which uses environment-controlled growth chambers that can accelerate plant development for research purposes. This includes phenotyping of adult plant traits, mutant studies and transformation, and the process has been demonstrated in wheat and other crops, thus offering great scope for crop improvement. The highlight of the speed breeding process is that it can reduce the generation time and shorten the breeding cycle, enabling rapid development of advanced stable lines and mapping populations, screening for identification of donor sources for trait(s) and faster development of improved cultivars in crops (Watson et al. 2018).

2.2 History of Speed Breeding

In earlier days, unpredictable climatic changes such as insufficient light during crop growth stages especially during winter season lead to reduced yields and crop failure in various parts of the world. In order to improve the low productivity and to prevent crop failure in these regions, the concept of growing plants in controlled environment with artificial light and in protected cultivation such as in greenhouses came into existence. Artificial lighting was used to supplement insufficient sunlight in protected cultivation in greenhouses and also in tissue culture laboratories where plant tissue cultures were maintained (Mpelkas 1980).

In the 1860s, the earliest references of using artificial electric lamps for plant growth and development were reported by Mangon (1861) and Prilleux (1869). Later Siemens (1880) used incandescent lamps and electric arc lamps in addition to natural sunlight for plant growth and development. Pfeiffer (1926) reported that artificial lighting period had significant influence on phyto-constituents of various plants. The utilization of light-emitting diodes (LEDs) especially blue diodes for

plant growth and development was demonstrated during the 1990s (Nakamura and Fasol 1997; Nakamura et al. 2000). During the same period, the National Aeronautics and Space Administration (NASA), USA, in association with Utah State University explored the possibilities of growing rapid cycling wheat in space station; this has led to the development of new dwarf variety 'USU-Apogee' (Bugbee and Koerner 1997).

This technique inspired plant scientists at the University of Queensland, the University of Sydney and the John Innes Centre, Australia, who have improved the technique and coined the term 'speed breeding'. This technique helped to grow wheat in space using continuous light, triggered early plant reproduction and hastened the process of breeding cycle (Watson et al. 2018). Speed breeding technique does not require in vitro conditions for growing plants. The plants can be grown in controlled growth chambers or greenhouses using optimal light intensity and quality, particular day length and temperature, which accelerates various physiological processes in plants especially photosynthesis and flowering, thus shortening the generation time.

2.3 Methods and Application of Speed Breeding in Various Crops

Speed breeding can be used to shorten generation time and to accelerate crop breeding and research programmes including development of mapping populations, phenotyping adult plant traits, hasten backcrossing and pyramiding of traits, mutant studies and transformation (Watson et al. 2018). Speed breeding as a platform can be integrated with several other technologies such as high-throughput phenotyping and genotyping, marker-assisted selection, genomic selection, CRISPR gene editing, etc. and can accelerate the rate of crop improvement in many crops across the world. Different methods or protocols have been followed in many crops to shorten the generation time (Table 2.1), and the details are discussed below.

2.4 Speed Breeding in Cereals

The concept of speed breeding method was first established and demonstrated in cereal species, especially wheat. The original approach was first developed on working grain dormancy trait of wheat grown under controlled environmental condition (Hickey et al. 2009). Later inspired by the efforts of NASA, USA, to grow crops in space, experiments in controlled environment were carried out in spring bread wheat (*Triticum aestivum*), durum wheat (*Triticum durum*), barley (*Hordeum vulgare*) and the model grass *Brachypodium distachyon*, by following three speed breeding methods: speed breeding 1 (controlled environment chamber speed breeding conditions), speed breeding 2 (glasshouse speed breeding conditions) and speed breeding 3 (homemade growth room design for low-cost speed breeding) (Watson et al. 2018).

Table 2.1 Accelerated generation time and protocol of different crop species

Sl. no.	Crop	Method	Duration in field or uncontrolled greenhouse	Duration under accelerated generation breeding	References
1	Bread wheat	Speed breeding 1	105	62	Watson et al. (2018)
		Speed breeding 2	87	65	Watson et al. (2018)
2	Durum wheat	Speed breeding 1	102	62	Watson et al. (2018)
3	Barley	Speed breeding 1	102–115	55–60	Watson et al. (2018)
		Speed breeding 2	132	68	Watson et al. (2018)
4	Rice	Biotronbreeding system	122	80	Ohnishi et al. (2011)
		Modified controlled biotron speed breeding system	135	100	Rana et al. (2019)
		Rapid generation advance (RGA)	110–121	95–105	Collard et al. (2017)
5	Sorghum	In vitro plus in vivo	90–140	80	Rizal et al. (2014)
6	*B. distachyon*	Speed breeding 2	73	48	Watson et al. (2018)
7	*Medicago truncatula*		90–180	78–80	Watson et al. (2018)
8	Grass pea	Greenhouse	150–180	94–120	Ochatt et al. (2002)
9	Pea	In vitro plus in vivo	143	67–76	Ochatt et al. (2002)
		In vitro only	150–180	112	Espósito et al. (2012)
		In vitro plus in vivo	143	67–76	Espósito et al. (2012)
		Speed breeding 1	84	51	Watson et al. (2018)
10	Canola	Speed breeding 2	171	98	Watson et al. (2018)
11	Chickpea	Speed breeding 2	115	82	Watson et al. (2018)
12	*Amaranthus*	Speed breeding 1	70–120	NA	Stetter et al. (2016)
13	Soybean	Greenhouse	102–132	70	Nagatoshi and Fujita (2019)

(continued)

Table 2.1 (continued)

Sl. no.	Crop	Method	Duration in field or uncontrolled greenhouse	Duration under accelerated generation breeding	References
14	Bambara groundnut	Greenhouse	160	160	Ochatt et al. (2002)
		In vitro plus in vivo	160	125 (unpeeled), 110 (peeled seeds)	Ochatt et al. (2002)
15	Peanut	GreenhouseRGT	140–145	89–113	O'Connor et al. (2013)
16	Lentil	In vitro	80–110	45–46	Mobini et al. (2015)

1. Speed Breeding 1: This method consists of controlled environment chamber (Conviron BDW) where continuous light supply was set for 22 h for photoperiod. The light and temperature were set to ramp up and down for 1 h and 30 min to create a situation that mimic natural dawn and dusk conditions. The temperature was set at 22 °C during the photoperiod and 17 °C during the 2 h dark period. Humidity was set to 70%. Varied light intensity was adjusted with respect to wheat wherein the intensity was 360–380 μmol m^{-2} s^{-1} (highest value after ramping) during vegetative stage and 490–500 μmol m^{-2} s^{-1} (highest value after ramping) at adult plant stage.
2. Speed Breeding 2: In this method, plants were grown in temperature-controlled glasshouse fitted with high-pressure sodium vapour lamps. The temperature was programmed to 17 °C during night and 22 °C during daytime and photoperiod duration for 22 h. The 2 h period without lamps operating during the night was set. Light intensity was maintained at 440–650 μmol m^{-2} s^{-1} at adult plant stage.
3. Speed Breeding 3: In this method, low-cost homemade growth room of 3 m × 3 m × 3 m insulated with sandwich panelling fitted with seven LB-8 LED light boxes and a 1.5 horsepower inverter split system domestic air conditioner. The lights were set to run for 12 h photoperiod (12 h of light and 12 h of darkness) for 4 weeks and then increased to an 18 h photoperiod (18 h of light and 6 h of darkness). To maintain the temperature, the air conditioner was set to run at 18 °C during darkness and 21 °C when the LED lights were on, with fluctuation being no more than ±1 °C. Varied light intensity was maintained ranging from 210 to 260 μmol m^{-2} s^{-1} and at 50 cm above the pot from 340 to 590 μmol m^{-2} s^{-1}. The lights were situated at a height of 140 cm above the bench. In this room, 90 pots (20.3 cm diameter and 5 L volume) can be grown. Automatic watering was also set and other humidity conditions were ambient.

In Norwich, UK, experiments were conducted during spring and early summer to compare the growth of plants grown in speed breeding method with plants grown in glasshouses with no supplementary light or heating. It was observed that the plants grown in speed breeding method progressed faster to anthesis (flowering) in approx-

imately half the time compared to glasshouse conditions. Irrespective of cultivar or line or accession, plants reached anthesis in 35–39 days in wheat, except Chinese spring, 37–38 days for barley and 26 days in *Brachypodium distachyon*; at the same time, the corresponding glasshouse plants had only reached the early stem elongation or three-leaf growth stage, respectively. It was also observed that the time taken for anthesis was more or less uniform within each species under speed breeding conditions (Watson et al. 2018).

The following observations were noticed in speed breeding-grown plants compared to glasshouse with no supplementary light. In both crops, wheat and barley produced healthy number of spikelets per plant, but it was observed that the number of seeds per spike was less (not significantly) compared to glasshouse. The speed breeding-harvested seeds had comparable germination and viability as that of glasshouse-grown seeds, and the crossed seed from speed breeding-grown plants produced viable seeds in tetraploid and hexaploid wheat. In both wheat and barley, the plant growth and development was unaffected in the second generation. In order to apply the concept of speed breeding to screen and advance large breeding populations, a controlled glasshouse with supplementary lighting can be preferred over high-cost growth chambers (Watson et al. 2018).

Besides these, the cultivars of wheat and barley were phenotyped for key adult plant traits, and it was observed that the traits associated with ethyl methanesulfonate-induced mutation of the awn suppressor B1 locus and reduced height (Rht) genes in wheat could be accurately recapitulated under speed breeding conditions (Hoogendorn et al. 1988; Derkx et al. 2012). It was also observed that traits related to adult plant resistance in wheat leaf rust and wheat stripe rust can be scored accurately under speed breeding conditions (Hickey et al. 2012; Riaz et al. 2016). Recently, speed breeding method was also used for phenotyping multi-traits such as seminal root number, seminal root angle, tolerance to crown rot, resistance to leaf rust and plant height (Alahmad et al. 2018) to improve stay-green and root adaption in wheat (Christopher et al. 2015) and for incorporation of multiple disease resistance in barley (Hickey et al. 2017).

Application of speed breeding in different crops has varied responses when plants are exposed to photoperiods. The response of short-day plants such as rice and maize to speed breeding method was observed to be not as successful as compared to long-day and day-neutral plants, where short-day plants require photoperiods less than the critical day length for anthesis (Thomas and Vince-Prue 1996). However some exceptions were observed in short-day plants, where few species show facultative response to photoperiod. In long-day plants, the anthesis can be accelerated by extending the photoperiods because the critical day length of these plants also generally exceeded. Similarly in case of day-neutral plants, anthesis or flowering will occur regardless of the photoperiod.

In rice, initially biotron breeding system (Ohnishi et al. 2011) was used to shorten generation time by growing rice plants in artificial environmental chamber. In biotron breeding system, the rice plants were grown at 30 °C/25 °C (11 h day/13 h night) in a biotron. Light intensity was set at 350 μmol m^{-2} s^{-1}. Relative humidity

was regulated at 70%, and CO_2 level of 20% was supplied from a CO_2 gas cylinder and regulated by a biotron setting of 475 ppm. Later Rana et al. (2019) modified this protocol (modified controlled biotron speed breeding system). In this modified method, the rice plants were grown in a controlled growth chamber. Seeds were sterilized and incubated at 30 °C in the dark for 2 days and then placed on seedling nursery trays. Ten-day-old seedlings were transplanted in pots and were grown under a long day length (14/10 h light/dark) for 30 days to accelerate vegetative growth and then under a short day length (10/14 h light/dark) to accelerate reproductive development. The temperature was maintained at 30/25 °C light/dark. Relative humidity was set at 70%, and light intensity was set to 350 μmol m^{-2} s^{-1}. This method was successfully used to introgress salinity-tolerant gene 'hst1', from 'Kaijin' into high-yielding 'Yukinko-mai' rice variety, following single nucleotide polymorphism-based marker-assisted selection (Rana et al. 2019).

In recent years, rapid generation advance (RGA) technology has been used to shorten the generation cycle in rice (Collard et al. 2017). In RGA, as the name implies, the method can enable several generations or cycles (between F_2 and F_6 generations) within a short period of time compared to normal field conditions. The RGA method is similar to that of single seed descent (SSD) method where single seed from each plant is used to advance the generation by self-pollination to produce fixed or homozygous lines. The difference between RGA and SSD is that in RGA we manipulate growth conditions so as to enforce early flowering and seed set compared to normal field condition. RGA method has several advantages compared to other conventional breeding methods, as it requires less space and resources and many germplasm or lines can be screened in shorter time leading to saving money, time, etc. (Stoskopf et al. 1993; Poehlman and Sleper 1995).

In RGA two methods were followed to accelerate generation time in rice:

(a) Screen House RGA System: In this method, the rice seedlings were grown in trays of 8 rows × 13 columns (104 cells per tray; 36 cm × 56 cm), and each cell contained <40 cm^3 of soil for a single rice line. Direct dry seeding was performed in this system. Initially, 4–5 seeds were sown per cell to ensure germination rate, and plants were then thinned to one plant per cell in 10–14 days after seeding. As per the requirement, minimal fertilizer was applied.
(b) Field RGA System: In this method, rice seeds are sown in trays for seedling development. The seedlings trays were directly inserted into the soil to control plant growth by restricting root development and keep the plants smaller and with fewer tillers. Fencing was done to avoid damage from rodents. The plants were grown till they produce single panicle with sufficient seeds, and then it will be harvested. Efforts are being made in different parts of the world to further accelerate the generation time in rice which permits 4–5 generations per year instead of present day's 1–2 crops per year.

Sorghum is a model C_4 cereal crop possessing numerous traits that make it an excellent plant model for studying cereals, monocots, stress tolerance and C_4

species. Several traits that make it a model cereal include large and plenty of seeds, large embryos, ease for crossing, adapted to grow in a wide variety of soils and environments and medium-sized genome (~760 Mb). Though sorghum is a model species, it has some shortcomings like long-time breeding cycle, early desiccation of embryos from mutants, and uniculm nature in plants grown under controlled environments, making it difficult for their use in crossing to obtain selfed/crossed seeds. Rizal et al. (2014) have addressed two important aspects of sorghum: (1) production of cross- and self-pollinated seeds from the same plant and conservation of the vital embryos and (2) more importantly shortening of the breeding cycle. An elite cultivar BTx623 was used for producing mutant (M) populations using 0.28% ethyl methanesulfonate (BTx623 R) and gamma ray irradiation (BTx623 Z), and these were advanced to subsequent mutant generations (M2–M6). Further, landrace *Sorghum propinquum* along with other cultivars like IS 18551, IS 3620 and IS 40653 were used as polymorphic parents. BTx623 (R) and BTx623 (Z), were used as wild types. These populations were utilized in producing both cross and self-pollinated seeds from a single panicle. In spikelets after emasculation and crossing, an average of 100 sessile spikelets were left resulting in as many as 109 F_1 and up to 1000 self-pollinated seeds in a single panicle. This has eliminated the waiting period for generation, and seeds (both cross and self) obtained could be used for generation advancement and genetic analysis.

Seeds from most mutants and crossed seeds demonstrated premature desiccation leading to embryo degeneration. But, the desiccation was not observed when the immature embryos were grown in appropiate medium. Further, roots and shoots were produced within 12 h of germination in media. Ease to embryo rescue, the rate of germination and plant development were found to be best when the immature embryos isolated 10–12 days after pollination were utilized. The germination rates of selfed seeds and crossed seeds were 90–100% and 60–100%, respectively. The plantlets acclimatized for 1 week in YCS media after germination developed better shoots and roots and, hence, survived better than plantlets directly transplanted to soil immediately after taking away from solid MS medium. The survival rate of directly transplanted and YCS acclimatized plantlets was 80% and 100%, respectively. Five to ten F_2 immature embryos from each cross were germinated on MS medium 10–12 days after pollination for shortening the timeframe of obtaining F_2 plants. While the seeds left in the panicle were maturing, the seedlings from immature seeds in MS medium were about 1 month old. All F_1 immature seeds produced healthy plants, thereby producing F_2 seeds in less than 3 months. The direct germination of immature seeds also significantly reduced the time required for the next generation of seed production. Usually it takes 5–6 weeks more for mature seeds to produce plants. The embryo rescue method saved a minimum of 6 weeks per generation, resulting in saving of several months in a continuous breeding cycle. Therefore, the combination of these two described methods considerably shortened the life cycle.

2.5 Speed Breeding in Other Crops

In addition to cereals, speed breeding has been successfully used to shorten the generation time in other crops such as oilseeds, pulses, fruit trees, vegetables, vegetatively propagated crops, etc. Breeding for high yields in oilseeds is always a challenge for plant breeders, since it involves improving the seed yield and oil content simultaneously. Further, the traits are negatively correlated in most of the major oilseeds, for example, groundnut, rapeseed-mustard, soybean, sesame and sunflower. In addition, the photosensitivity and long crop cycle become hindrance for the breeders to hasten the breeding process. It has been stated that, in groundnut, releasing a variety takes 10–12 years in India, and this duration could exceed in subtropical and temperate countries. Similarly rapeseed-mustard and mustard can be grown in single season or two seasons per year, which increases the time for generation advancement to attain homozygosity.

The crop duration in soybean is 100–125 days, in which 50% of the length of life cycle is reproductive stage. The ripening of the pods takes longer duration. Nevertheless, soybean seed maturation studies demonstrated that seed maturation and germination ability of the embryo were independent of the seed mass, and the production of viable seeds is possible through precocious germination (Burris 1973). Taking the leads of possibility of precocious germination, Roumet and Morin (1997) demonstrated that pre-treatment of immature pods at 26 °C temperature for 4 days resulted in precocious germination in 22 genotypes representing broad genetic background. The immature pods sampled after 18 days after flowering showed 100% germination, and 73% developed into plantlets to produce viable seeds. Thus the growth cycle was reduced from 130–170 to 65–70 days. Recently, Nagatoshi and Fujita (2019) have standardized speed breeding protocol for elite Japanese soybean cultivar, Enrei. The soybean cultivar's crop duration was reduced to 70 from 102 to 132 days; consequently 5 generations per year were raised instead of 1–2 generations. The method utilizes commonly used fluorescent lamps (220 μmol m^{-2} s^{-1} at the canopy level), a 14 h light (30 °C)/10 h dark (25 °C) cycle and carbon dioxide (CO_2) supplementation at >400 ppm. This speed breeding protocol is useful as crossing nurseries and advancing generations.

In Canola, four generations per year were achieved by speed breeding in fully enclosed controlled environment growth chamber. The protocol developed by Watson et al. (2018) was not only for advancing the generations but also for phenotyping pod shattering trait of canola. Five cultivars of canola susceptible to pod shattering were grown under environment-controlled growth chambers. Pods from plants were harvested after 6 weeks post anthesis. These pods were dried in oven for 5 days at 35 °C. The seeds from these pods were threshed and used for germination test. Upon successful germination and plant establishment, irrigation was regulated by reducing water supply after 4–6 weeks depending on the flowering of the cultivar. Irrigation was reduced to every 2 days for 1 week followed by twice in a week for 1 week, and finally water supply was stopped after seventh and ninth week. The

plants were fully senesced, and pods were harvested and subjected to shattering test using pendulum pod shatter protocol (Liu et al. 1994).

Sunflower is a short-duration crop of 80–100 days depending on the cultivars. Nevertheless, seed dormancy of fresh harvest in sunflower limits the number of generations per year. Dormancy of fresh seed is one of the main hindrances for speed breeding. In sunflower, fresh seed dormancy prevails up to 40–70 days after harvest. Seed dormancy is a complex trait which is influenced by environment and endogenous factors. In sunflower, both seed coat-imposed dormancy and embryo dormancy exist. Corbineau et al. (1990) demonstrated the inability of fresh seed for germination at optimum temperature of 25 °C . Seed drying by storage at 5–40 °C temperature improved the germination rate. Germination rate improved by removing the pericarp from the stored seeds (30 days after harvest), thus indicating the role of pericarp in dormancy due to physical and chemical factors, inhibiting water absorption (Vigliocco et al. 2017). Seed treatment using hot water (80–100 °C), gibberellic acid, ethrel, potassium nitrate and thiourea are being used to break the seed dormancy in sunflower. But, these treatments are being used on the seeds stored for at least 30 days after harvest. There is a research gap for developing techniques to improve germination rate in fresh seeds. Once the germination of fresh seeds is established successfully, speed breeding in sunflower will be very successful for generation advancement and phenotyping of trait.

The short breeding cycle protocol for sunflower was developed by Dağüstü et al. (2012) using embryo rescue technique. Embryos from seeds set after 10–12 days after pollination were cultured in MS media (Murashige and Skoog 1962) with 2% sucrose and 0.8% agar at pH 5.6–5.7. The plantlets with well-developed roots and shoots were transferred to soil and maintained in controlled environment condition (24 ± 2 °C in 16 h/8 h (light/dark) in the growth chamber). In a total of 1320 plantlets, 70.3% developed into mature plants yielding seeds. The embryo rescue techniques are well standardized for sunflower which can be used to tackle fresh seed dormancy. Therefore integrating embryo rescue techniques, speed breeding for sunflower can be achieved to shorten the breeding programme.

Sesame is a potential oilseed crop which has high scope for speed breeding. It's a short-duration crop (70–100 days) and photo- and thermosensitive, with high level of genetic variability existing for improvement. Therefore, advancing generations using speed breeding is advantageous for improvement of several agronomic characters which needs immediate attention such as shattering and indeterminate growth habit. The crop is highly susceptible for fungi and phytoplasma, due to which productivity is under stake. Breeding efforts on development of disease-free, phytoplasma-free sesame are in top priority among sesame breeders across the globe. Standardization of speed breeding techniques under controlled environmental conditions for sesame crop is yet to be taken up, and it has high scope for success.

The ever-increasing population has increased the demand for plant proteins as animal feed and for human consumption. This has led to the development of protein-rich seed legumes in several pulses like soybean, pea, grass pea, Bambara groundnut and others (Campbell 1997; Heller et al. 1997). The main bottleneck in any breeding programmes is the generation time that it takes for advancing the generations. Hence, it

is important to shorten each generation cycle by hastening flowering and seed set. Legumes are regarded as recalcitrant to in vitro approaches (Ochatt et al. 2002) which lead to production of sterile plants or plants with reduced fertility (Bean et al. 1997). Though greenhouse technology has been the easiest way of shortening the generation cycle, there are other methodologies like in vitro only strategy and combination of in vitro and in vivo strategy to reduce the generation time. Below we discuss three methodologies followed for rapid generation advancement (RGA) in pulses.

1. Greenhouse: This type of speed breeding technologies involves growing plants in controlled environmental conditions (CEnvC), with controlled light and temperature conditions in a greenhouse environment.
2. In vitro only strategy: Here all stages and including seed set occur in vitro. This involves growing plants in artificial medium and allowing them to produce a few flowers which develop seeds that are harvested prior to normal seed maturity. In vitro protocols for RGA were successfully developed for pea (*Pisum sativum* L.), lentil (*Lens culinaris* Medikus), chickpea (*Cicer arietinum* L.), common bean (*Phaseolus vulgaris* L.) and faba bean (*Vicia faba* L.). In vitro RGA resulted in approximately 90% of plants setting seed, which was sufficient for biotic or abiotic stress tolerance screening. The high cost of labour and materials limits the usefulness of in vitro RGT for pulse crop breeding and research.
3. In vitro plus in vivo strategy: This is an intermediate methodology where embryo rescue is not involved when compared to in vitro only strategy

Ochatt et al. (2002) developed a greenhouse RGT for pea and Bambara groundnut wherein seeds were sown in 38-well tray at the rate of 230 plants/m^2 using perlite as growth substance and nourished by capillarity with a nutrient solution (14.44 mM NO_3, 3.94 mM NH_4, 15.88 mM Ca, 17.9 mM K_2O, 4 mM MgO, 2.46 mM P_2O_5, 2.00 mM SO_3 and various microelements). For pea, temperature was controlled at 20 °C/16 °C day/night, with a maximum of 26 °C. In order to reduce internode elongation, anti-gibberellin (flurprimidol 2-methyl-1-pyrimidine-5-yl-1-(4-trifluoromethoxyphenyl)propane-1-ol) (Topflor, Dow AgroSciences, France) was used (0.5% w/v). Anti-gibberellin spar was done at three-leaf stage and further two more sprays at 10-day interval. Rapid maturation was obtained by stopping watering and nourishing when pods were whitish (50–60% seed dry matter content), the perlite substrate favouring plant dehydration. Harvesting was done by hand at full maturity to preserve maximum germination, and seeds were re-sown immediately following the same procedure. For Bambara groundnut, temperature was controlled at 27 ± 1 °C/25 ± 1 °C day/night, and 10 h light photoperiod of about 5000 lux was used.

In addition to greenhouse RGT, Ochatt et al. (2002) also developed an in vitro plus in vivo strategy of RGT for pea and Bambara groundnut. In this process, first sowing was done using seeds which were imbibed overnight and sown in container with vermiculite imbibed first with nutrient solution (14.44 mM NO_3, 3.94 mM NH_4, 15.88 mM Ca, 17.9 mM K_2O, 4 mM MgO, 2.46 mM P_2O_5, 2.00 mM SO_3 and various microelements) and then with deionized water. For pea, after sowing, plants were kept under a 16 h/8 h light/dark photoperiod at 24 °C/20 °C, with around 70% constant

relative humidity. At around 60 days, yellowing pods with mature non-dry seeds were harvested and used for raising next generation. For raising next generation, non-dry seeds were surface-sterilized by successive dips into ethanol 70% (1 min) and $Ca(ClO)_2$ at 35 g/L (20 min). Then pods are shelled under aseptic condition and three central seeds to be used for sowing. Outer and inner integuments are opened without damaging cotyledon and embryo axes. Embryos were then cultured on solid agar medium with half-strength media (Murashige and Skoog 1962) with macro-elements, full-strength MS media with microelements, Fe-ethylenediaminetetraacetic acid (EDTA) and MS vitamins, plus 15 g/L sucrose (pH 6). Media were poured either into transparent plastic pots with 30 mL medium and loosely closed to favour gas exchange, into Petri dishes with 20 mL medium or into culture tubes. They were kept at 24 °C/22 °C, under a 16 h/8 h (light/dark) photoperiod. At around 2–3 weeks when plants were 4–5 cm tall, they were transferred to growth chamber into large container containing vermiculite kept until new pods were mature enough for the extraction of the next seed generation. For Bambara groundnut after sowing, plants were kept under a 16 h/8 h light/dark photoperiod at 24 °C/20 °C, with around 70% constant relative humidity. At around 60 days, yellowing pods with mature non-dry seeds were harvested and used for raising next generation. For raising next generation, non-dry seeds were surface-sterilized by successive dips into ethanol 70% (1 min) and $Ca(ClO)_2$ at 50 g/L (30 min). Then pods are shelled under aseptic condition and used for sowing. Embryos were then cultured on solid agar medium with MS macro-elements, microelements and vitamins of Nitsch and Nitsch (1969), with 2% sucrose, plus different types and concentrations of phytohormones. For seed germination, only half-strength BM medium was used. Media were poured either into transparent plastic pots with 30 mL medium and loosely closed to favour gas exchange, into Petri dishes with 20 mL medium or into culture tubes. They were kept at 24 °C/22 °C, under a 10 h light photoperiod of about 5000 lux. At around 2–3 weeks when plants are 3–4 cm tall, they are transferred to growth chamber into large container containing vermiculite kept until new pods were mature enough for the extraction of the next seed generation**.**

Apart from greenhouse RGT and combination of in vitro and in vivo strategy, Espósito et al. (2012) developed an in vitro only strategy for RGT of pea. Here seeds of the pea varieties were surface-sterilized in 70% ethanol for 1 min, subsequently were immersed in 25% commercial sodium hypochlorite solution for 10 min and were rinsed twice in sterile water. Seeds are to be cultured on basic medium MS (Murashige and Skoog 1962) + B5 vitamins + 25 μM BA + 30 g/L sucrose + 6 g/L agar and pH adjusted to 5.7. After 2–3 weeks, shoots were transferred into rooting media containing MS medium supplemented with 0.01 mg/L α-naphthaleneacetic acid. All cultures were incubated in a growth chamber at a temperature of 24 °C, a photoperiod of 16 h and 40 mM m^{-2} s^{-1} irradiance. At around 6 weeks after rooting, plantlets were then transplanted to normal greenhouse potting soil and grown to maturity in the greenhouse. Approximately, 60% of regenerated shoots produced roots in the culture media.

O'Connor et al. (2013) proposed a greenhouse RGT for peanut which involved growing peanuts in 30 cm pots under greenhouse conditions with continuous light

(24 h) provided by 450 W photosynthetically active radiation (PAR) lamps. The potting media consisted of two layers, a lower section of two parts krasnozem (sourced from the A horizon of a field trial plot) to one part alluvial sand and an upper section, 50 cm in depth, of pasteurized peat mix consisting of nine parts alluvial sand, six parts peat moss and one part krasnozem. A granular nitrogen, phosphorus and potassium basal fertilizer needs to be applied to the peat mix as basal dose before sowing, and pots need to be watered up to field capacity. Recommended plant population is ten plants per pot with 32 °C/22 °C maximum/minimum temperature and 65% relative humidity. With the use of continuous light in combination with optimum temperature and humidity in the greenhouse facility, O'Connor et al. (2013) observed considerable increase in the rate of plant development compared to field conditions. The parental lines, Farnsfield and D147-p3-115, which usually take 140–145 days to reach full maturity under field conditions, took 113 and 89 days in F_2 and F_3 generations, respectively, using speed breeding techniques. From the initial 400 seeds planted in the F_2 generation, there was a 68% seed recovery with 270 plants producing viable F_3 seeds available for planting. From the F_3 seed source, there was a 74% recovery rate, with a total of 201 viable F_4 seeds harvested. Further research is required to understand the reasons behind plant loss and to recover 100% viable seeds.

In vitro RGT of lentil (Mobini et al. 2015) involves growing plants in artificial medium and allowing them to produce a few flowers. The developed seeds were then harvested prior to normal seed maturity. Mobini et al. (2015) developed an in vitro RGT protocol for lentil in which they compared three different in vitro methods, i.e. (1) tissue culture method using agar as substrate and MS as medium, (2) hydroponic system using perlite as substrate and HS as medium and (3) agar as substrate and HS as medium. Among medium (i.e. HS vs. MS), using HS compared to 1/2 MS medium accelerated flowering time by 3.5 days, and flowering and seed setting rates significantly increased, by 21.2% and 11.2%, respectively. Among substrates (agar vs. perlite), perlite significantly increased flowering and seed setting rates by 18.4% and 19.1%, respectively, and resulted in an average of 4.7 fewer days to flower initiation. Mature lentil seeds were washed using Tween 80 (0.1 mL/L) for 4 min, and later seeds were surface-sterilized by immersion in $Ca(ClO)_2$ at 15 g/L for 20 min and then rinsed two to three times in sterile water and soaked for 8 h. Then seeds were completely drained and kept in an incubator (24 °C) in darkness overnight. The following day, seeds were washed with sterile water another two to three times, drained and returned to the incubator. After emergence of the root and shoot, which took place within 2–3 days, seedlings were transferred to an incubator with 22 °C/18 °C day/night temperature and 178 µmol m^{-2} s^{-1} light intensity with 18 h light/6 h darkness. The germinating seeds were then cultured for 3 days on medium solidified with agar, consisting of modified MS medium with half-strength ammonium nitrate and MS microelements, plus 30 g/L sucrose (pH 6.5), 0.05 µM 4-Cl-IAA and 0.9 µM flurprimidol. The plants were then cultured in double Magenta™ boxes with 19 cm height. The temperature was controlled at 20 °C/18 °C day/night, with 20 h light and 4 h darkness. Once seeds approached physiological maturity (12–14 days after flowering), they were separated from the pods and trans-

ferred to modified B5 medium (Gamborg et al. 1968) in order to facilitate embryo rescue and germination in sterile conditions. Light source: T5 (841, Philips, Amsterdam, The Netherlands) cool white fluorescent tubes with PAR at 500 μmol s^{-1} m^{-2} and red (655–665 nm) to far-red (725–735 nm) ratio of 1.9 were used as light source. The red to far-red ratio was assessed using a radio spectrometer (Apogee, PS-200, Logan, UT). Using this in vitro RGT, lentils flowered by 31–33 days and reached physiological maturity by 45–46 days. These immature seeds were then advanced to next generation by using embryo rescue and thus completed one generation in 46 days. Under these conditions, 94.3% of all plants flowered, and 81.2% of all plants set seeds. It may be possible to produce eight generations of lentil within 1 year using this approach. In contrast, only three generations per year are possible in the growth chamber or greenhouse using the conventional single seed descent (SSD) method.

Mobini et al. (2015) extended the in vitro RGT of lentil to faba bean with slight modifications. They compared the effect of tissue culture (ChM1 medium with agar), hydroponic method (HS with perlite) and intermediate method (HS with agar) combined with two different auxins (IAA and 4-Cl-IAA) with or without zeatin for days to flowering and flowering and seed set rates. There was no significant difference between ChM1 and HS, and agar and perlite, for all three parameters. Tissue culture, hydroponics and intermediate methods were not significantly different among treatments. However, the combination of 5.7 μM IAA and 2.3 μM zeatin in combination with the intermediate method (HS with agar) led to 4.9 days earlier flowering and also increased flowering and seed setting rates by 28.4% and 56.0%, respectively, compared to all other treatments. Mature faba bean seeds were washed using Tween 80 (0.1 mL/L) for 4 min before sterilization. Seeds were surface-sterilized by immersion in $Ca(ClO)_2$ at 15 g/L for 20 min and then rinsed two to three times in sterile water and soaked for 8 h. Then seeds were completely drained and kept in an incubator (24 °C) in darkness overnight. The following day, seeds were washed with sterile water another two to three times, drained and returned to the incubator. After emergence of the root and shoot, which took place within 6–7 days for faba bean, seedlings were transferred to an incubator with 22 °C /18 °C day/night temperature and 178 μmol m^{-2} s^{-1} light intensity with 18 h light/6 h darkness. The germinating seeds were then cultured for 3 days on medium solidified with agar (A7002, Sigma-Aldrich Corp., St. Louis, MO), consisting of modified ChM1 medium with half-strength ammonium nitrate and MS microelements, plus 40 g/L sucrose (pH 6.5). The plants were then cultured in double Magenta™ boxes with 19 cm height. The temperature was controlled at 20 °C /18 °C day/night, with 20 h light and 4 h darkness. Once seeds approached physiological maturity (17–18 days after flowering), they were separated from the pods and transferred to modified B5 medium (Gamborg et al. 1968) in order to facilitate germination in sterile conditions. Since faba bean seeds are relatively large, the time required for germination of faba bean was greater than lentil, while seedling growth rate was faster. Under optimal conditions with the addition of PGRs (5.7 μM IAA + 2.3 μM zeatin + 0.3 μM flurprimidol) and use of

HS medium, a mean of 32 days to flowering was achieved in both cultivars. The generation time was 54 days, including 18 days required for immature seeds to be ready for embryo rescue. Using in vitro flowering and RGT, 6.8 generations per year could potentially be produced for faba bean, compared to 1 generation per year in the field and 3 in the greenhouse.

In majority of fruit crops, flowering usually occurs after the juvenile phase. The juvenile phase in fruit crops has a wide duration ranging from a few years to >20 years (Korbo et al. 2013). Therefore, juvenile phase is mostly targeted to reduce and accelerate the breeding cycle. Some reports that promote vigorous vegetative growth resulting in early flowering compared to the normal time include (1) apple (*Malus* × *domestica*), 10 months compared to 5 years (Van Nocker and Gardiner 2014), and (2) chestnut (*Castanea sativa*), 2 years instead of 7 (Baier et al. 2012). Extra resources and protocol modifications to aid in efficiently managing the plants under controlled conditions are the major challenges in speed breeding of tree crops.

Apple (*Malus x domestica* Borkh.) breeding is time-consuming because of prolonged breeding cycles, requiring substantial field space and labour-intensive and involves substantial expenditure. The development of a new apple cultivar takes ~15–20 years; more time is needed when a desirable trait has to be introduced from wild species. Removal of unwanted alleles (linkage drag) from wild species requires ≥5 generation cycles (Joshi et al. 2009). Each generation cycle ranges between 4 and 10 years based on the genetic background and cultivation technology of seedling (Flachowsky et al. 2009). Therefore, it takes ~50 years or more to develop a new cultivar with desirable trait from wild species and good fruit quality (Schouten et al. 2006). Flachowsky et al. (2011) have reported successful application of speed breeding technology to apple based on transgenic early-flowering plants and marker-assisted selection. Twenty-four *BpMADS4* transgenic apple lines developed from the German cv 'Pinova' were evaluated under long-day conditions (16 h light, 8 h darkness) at 22 °C. At flower initiation, T1165, T1187 and T1190 lines were placed in glasshouse conditions with no additional light, and during winter (January–March), 'artificial winter' conditions were applied by reducing the temperature from 3 to 0 °C from 1 h before to 9 h after sunrise. But, from April to December, the plants were grown under glasshouse conditions similar to those of natural orchard conditions. The 24 *BpMADS4* transgenic lines flowered within <10 months after rooting and indicate the importance of *BpMADS4* gene to reduce the juvenile phase of apple. A breeding programme was started by crossing early-flowering T1190 with the fire blight-resistant *Malus fusca*. Early-flowering transgenic F_1 seedlings were identified and backcrossed with 'Regia' and 98/6–10 to introgress the apple scab (Rvi2 and Rvi4) and powdery mildew (Pl-1 and Pl-2) resistance genes along with FB-B7, fire blight resistance QTL in 'Regia'. Three transgenic BC_1seedlings with scab gene (Rvi2, Rvi4) and fire blight QTL (FB-F7) and also three other BC_1 seedlings combining powdery mildew resistance genes (Pl-1 and Pl-2) were identified.

Speed breeding could help in stimulating early flowering in late flowering or recalcitrant lines in leafy vegetable crops. Further, speed breeding could aid in synchronized flowering in varied germplasm lines used in vegetable breeding. Development of new varieties is required especially in orphan leafy vegetable crops which are still in the process of being domesticated or at early stages. Pest/disease resistance and uniformity in the harvested foliage and other products are the major breeding objectives for leafy vegetables. Some of these objectives have already been achieved through a speed breeding protocol developed in amaranth (Stetter et al. 2016). Grain amaranth has a field generation time of about 6 months during which the plants become very tall with thousands of small flowers. Short-day conditions (8 h) coupled with high temperature (30 °C) induced flowering in 4 weeks compared to the usual 10 weeks. Further, flower initiation could be controlled under short-day conditions. Usually, under long-day conditions (16 h, 35 °C), the plants exhibit large vegetative growth and late flowering (~10 weeks). The transfer of plants to short-day conditions aids in synchronous flowering for crossing between genotypes that exhibit different flowering behaviour. The short-day treatment helps in production of large amounts of pollen when the female plants start flowering, and after 4 weeks of flowering, mature seeds could be harvested. The modified growing conditions result in very short generation time that allows up to six generations per year, comparable to *Arabidopsis thaliana*. Additionally, adjusting growing conditions can help in controlling plant height and seed number per plant.

Clonally propagated crops like tubers, banana and roots have limited diversity, and mostly researchers and consumers are dependent only on few clones (Heslop-Harrison and Schwarzacher 2007) making them extremely vulnerable to disease and pest epidemics. Speed breeding techniques will aid in introduction of desirable traits in shorter timeframe, particularly for diseases like bacterial wilt in banana and brown streak in cassava. Initiation of crossing programme is a real challenge in these vegetatively propagated crops as the flowering is late, rare or under special conditions. Some speed breeding efforts have been initiated to shorten the time for flowering and increase the flowering rate along with predictability of many species (Wilson 1979; Jamnadass et al. 2015; Ceballos et al. 2017; Silva Souza et al. 2018).

To conclude, modern breeding programmes should be at par with the changing climate, reducing the breeding cycle to develop climate-resilient crops is need of the hour which can be accomplished by new ideas like speed breeding. Speed breeding has been used by many researchers to develop crops in shorter duration. Speed breeding can be integrated with other breeding techniques such as single seed descent method, high-throughput phenotyping and genotyping, etc. to accelerate the generation time in plant breeding programmes. Genomics-assisted breeding can be utilized to estimate the breeding value of individual plants, and then accelerated breeding protocols can enable faster crossing programme and shorten the growth duration cycle. An integrated approach by utilizing speed breeding helps in deveopment of new well-adapted cultivars in a shortest possible time to meet the future demand.

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Chapter 3
Genomic Selection in Cereal Crops: Methods and Applications

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Abbreviations

AFLP	Amplified fragment length polymorphism
ARMS	Amplification refractory mutation system
BLUE	Best linear unbiased estimation
BLUP	Best linear unbiased prediction
CIMMIYT	International Maize and Wheat Improvement Center
CRISPR	Clustered regularly interspaced short palindromic repeats
DArT	Diversity arrays technology
DH	Doubled haploids
F1	First filial generation
GBLUP	Genomic best linear unbiased prediction
GEBVs	Genomic estimated breeding values
GS	Genomic selection
HTG	High-throughput genotyping
IRGAs	Infrared gas analyzers
IRT	Infrared thermography
ISSR	Inter-simple sequence repeats
LASSO	Least absolute shrinkage and selection operator
LD	Linkage disequilibrium
LS	Least square
MAS	Marker-assisted selection
MCMC	Markov chain Monte Carlo
MTA	Marker-trait association
NAM	Nested association mapping

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NCII	North Carolina II design
*N*e	Effective population size
NGS	Next-generation sequencing
PLS	Partial least square
PM-RKHS	Pedigree plus molecular marker model using reproducing kernel Hilbert space regression
QTL	Quantitative trait loci
RBFNN	Radial basis function neural networks
RF	Random forest
RFLP	Restriction fragment length polymorphism
RILs	Recombinant inbred lines
RTK-GPS	Global position system-real-time kinematic
RR	Ridge regression
SNPs	Single nucleotide polymorphisms
SSRs	Simple sequence repeats
STS	Sequence-tagged site
SVM	Support vector machine
TRAPs	Target region amplification polymorphisms
TRN	Training population
TST	Testing population
WBSR	Weight Bayesian shrinkage regression

3.1 Introduction

In contemporary plant science research, we have been getting a wide fluctuation in data due to global environment effect (Crossa et al. 2017). It is affected by various abiotic conditions such as rainfall patterns, temperature, humidity, and snowfall. The drastic changes in the important agronomic parameters have also reduced the yield worldwide. During the twentieth century, genetic improvement was carried out using phenotypic selection and pedigree analysis. But sometimes it was not moving toward higher grain yield in drought-stressed and semi-arid region. In the twenty-first century, it is possible to resolve the demand of high crop yields using advanced genomic approaches such as genomic selection (GS).

Genomic selection is the combined approaches of advanced traditional breeding, development of double haploid (DH) lines, concept of field design, and quantitative population genetics integration in various cereal crops. GS with molecular tool pushed more genetic gain particularly over marker-assisted selection (MAS) for background and foreground selection, identification of variant alleles, and multiple cycle selection by crossing (Bassi et al. 2016). The major advantage of this technology is reduced number of genetic markers obtained at population generation advancement of field study. Flanking genes with single marker analysis by

genome-wide high-throughput marker is more efficient than genome-wide background selection by high-throughput marker (Bassi et al. 2016). It is possible that sometimes due to lack of marker availability and nature of trait, it has not lead to genetic gain.

With the huge success of GS in animal breeding technology, its use is now extended to plants specially crops. Human acquires two-thirds of the total daily energy intake from crop plants (Smit et al. 1999). It is observed that due to increasing human population, breeders are forced to develop crop varieties having higher yield. Several assessments have been done and concluded that we can increase genetic gain more rapidly by GS than traditional breeding. Thus, several programs have been implemented to get the best strategy of GS and how can we provide good guidelines based on good agricultural practices. This chapter provides statistical model and their concept with key consideration in GS breeding approaches. Furthermore, this chapter would be helpful to design prime breeding schemes for GS, select training (TRP) and breeding population (BP), estimate breeding value, thus help in reducing the gap between production and demand of rising world population by decreasing the rate of per unit time and cost in genetic gain.

In GS breeding program, the population used for both phenotype and genotype is called training population (TRP), while the population having only genotype is called breeding population (BP) or test population (TSP). The BP is used to identify loci associated with the trait in TRP. Thus, population size, nature of TRP, and relatedness of TRP to the BP are the main factors affecting GS efficacy. The rationale of GS is to maintain the accurate phenotypic selection besides exhaustive molecular genetic approaches. GS in crop plants is quite different from animal livestock breeding (Shamshad and Sharma 2018). Nevertheless, this chapter illuminates the power and application of GS based on high-density marker platform. However, the efficacy and power of GS depend on the genomic architecture of the crop plant. MAS-based linkage map is unable to capture the minor gene effect, so it is difficult to use it for traits having complex inheritance.

Traditional breeding takes too much time in the selection of elite lines. The major advantage of GS over traditional selection is that it can enhance the grain yield more rapidly by reducing the breeding cycle interval. Variety selected based on genomic selection has shown better adaptation to climatic changes. Thus, GS facilitate expeditious selection of superior variety/cultivar in less time by reducing breeding cycle. In this chapter, we discuss the history, basis, advantages, and many more aspect of GS. We also learn the statistical model and concept of GS and what are the new techniques used in genomic selection. Since the last two decades, a huge number of markers were identified and developed in crop plants and are available for molecular breeding. Recently, the most prominent marker used in genotyping is single nucleotide polymorphisms (SNPs), in general high-throughput genotyping (HTG). SNPs are intensively used for the identification of QTLs or genes associated with the trait using linkage mapping and association mapping. Bi-parental population is used in linkage analysis but has low power of detection due to limited genetic recombination. To overcome the low power of linkage analysis, diverse population

is used to identify QTL by association mapping approaches (Rahim et al. 2018). Thus, marker-trait association (MTA) with high rate of recombination is able to identify the minor-effect variants which are associated with economically important traits.

From statistical model, important relationships between phenotypes and genotypes of breeding population is predicted based on training population, wherein the statistical equation used in genomic selection is known as genomic selection models. The efficacy of statistical model is determined based on the available data of both genotypes and phenotypes. Higher number of individuals and marker density makes the predictions more powerful in training population. The main concern of phenotype data is to predict marker effect, and then the genotype data is put into the model to predict GEBV. The accuracy of selection depends on the response of GEBV, additionally on heritability, nature of traits, effective population size, and pedigree involved in the selection. Based on the encouraging results obtained in some major cereals, preliminary measures were taken to deploy GS to develop superior lines more rapidly and enhance the genetic gain rate in some legume crops, like groundnut, pea, soybean, chickpea, and pigeon pea (Varshney 2016). Software and tools developed for automated imaging have enabled capturing of phenotype data at high speed and greater volumes. Such technologies are global position system-real-time kinematic, infrared thermography, infrared gas analyzers, etc. which give efficient measurement of traits across plant studies. Thus, high-throughput phenotyping decreases the burden on phenotype analysis, and the accuracy in data reflects the true breeding value.

The introduction of NGS technologies has revolutionized genomic pipeline to be adopted for high-throughput screening of populations which has very important role in MAS, GWAS, and genomic selection. Today, NGS platforms and genotyping protocols are being designed for many cereal crops, which help in cost-effective genotyping of association panels and mapping populations with hundreds to thousands of markers (Rasheed et al. 2017) and lead to the discovery of a large number of molecular markers. GS is an organized and managed breeding effort for various crops in reaping the advantage of cutting-edge research in biological sciences with combination of classical phenotype-based approaches (Varshney and Tuberosa 2007).

The major objective of crop improvement worldwide is to develop high-yielding varieties. QTL/gene identification that is responsible for a specific trait is one of the main concerns in GS. After the identification of candidate gene from a QTL, different molecular techniques such as sequence analysis, gene expression analysis, gene cloning, gene transformation, etc. can be implemented to further study the molecular basis and functional validation of specific gene of interest. With the development of marker technique and dense DNA marker availability, there is another potential benefit of use of genomics tools in breeding program followed by map-based cloning of important genes or QTL.

3.2 Backgrounds

3.2.1 Breeding Selection

Mating, statistical test, and selection are the basics of the breeding in population genetics. The term selection is very commonly used in many fields for different types of elements. In breeding sciences, the animal's breed, plant's varieties, and races are used as biological element.

In 1957, Breese and Mather described the natural selection on genetic architecture of *Drosophila melanogaster* and described the effect of polygenic systems in natural selection. They studied how the dual systems of interactions (dominance and epistasis) carry out toward the selection. It is clear that the combination of gene interaction in species is the basis of genetic architecture which differs from trait to trait, governed by small- and main-effect genes.

In breeding programs, both types of selection, i.e., natural and artificial selection, are found (Herrendorfer and Tuchscherer 1996). The natural selection is not influenced by human individual. Breeding selection is done to define for selection of the best line from a given population. Indeed for the precision of artificial breeding programs, the information of each cross is required.

3.2.2 Marker-Based Selection

The development of new variety or breed by conventional breeding is a long process. To overcome this problem, a new breeding scheme has been developed as marker-assisted selection (MAS), which is a more efficient approach for the selection of desirable varieties or traits based on the associated molecular markers (SSRs, AFLP, RFLP, ISSR, SNPs, SCAR, RAPD). The quality of markers such as ease of use, low cost, repeatability of result, highly polymorphic, distribution throughout the genome, and co-dominance is required for the efficiency of MAS. In conventional breeding, recessive allele related to the trait is identified by selfing or test crossing, and this step is not required in MAS. MAS provides more progressive result than phenotypic selection because it uses a combination of phenotypic data and molecular markers; however, the efficiency depends on the heritability of the trait (Lande and Thompson). Other factors that affect the best association of marker or quantitative loci to the traits are sampling errors, statistical tests, scoring of markers, and phenotypic information. Thus, marker-based selection has revolutionized the varietal development when integrated with the traditional breeding. Selection of parent having contrasting trait is an important step in MAS (Fig. 3.1). Developments of breeding population, extraction of genomic DNA, fine scoring of markers, and correct correlation of phenotype with marker are important steps in MAS.

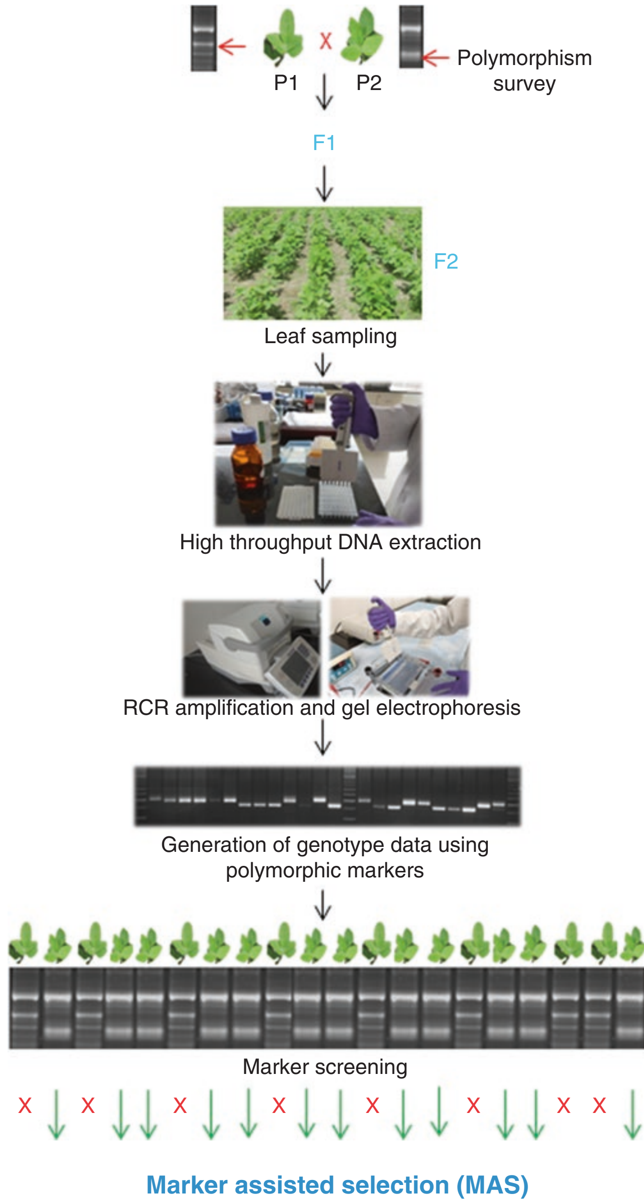

Fig. 3.1 Graphical representation of basic steps of marker-assisted selection strategy for crop improvement

3.2.3 Genomic Selection

3.2.3.1 What Is Genomic Selection?

GS is a robust approach to enhance genetic potential of the crops. The basic idea behind GS approach is to use genome-wide marker data to effectively select elite individuals with superior traits. GS makes use of genome-wide markers such as SNPs to predict genetic architecture of the training population with subsequent selection of breeding individual with superior phenotype. It uses phenotype and genotype data of past trials of lines/test population or breeding population to predict the breeding values of individuals that have not been phenotyped, thus reducing the time required for screening superior individuals for breeding.

3.2.3.2 How GS Works?

For GS, extensively phenotyped training population which is genotyped using genome-wide markers such as SNPs is prerequisite. A suitable statistical model showing association of genetic loci with phenotypes is created to estimate genomic estimated breeding values (GEBVs). The breeding value of non-phenotyped individuals (breeding population) is predicted based on its genetic relationship determined from the statistical model developed with the training population. Individuals from breeding population can be selected based on their breeding values for breeding purpose to speed up genetic gain. Figure 3.2 explained the selection of TRP that was phenotyped and genotyped in the first phase and used for model prediction in the second phase and update of predicted model at up to five successive generations for the accuracy of model. This can be derived from the base population with random selection of effective population size (N = 30–60). The third phase start from the breeding population developed with best parent based on the highest GEBV of desired trait. These breeding population are genotyped, and the previously predicted model is used for the evaluation of phenotype and to pick the highest ranked genotyping value seedling for the development of elite line. In the fourth phase, we have the complete knowledge of phenotype and genotype that was tested and verified for field plantation and clonal propagation.

3.2.4 Importance of GS

Complex quantitative traits are polygenic, i.e., multiple genes/locus regulates a trait. One key advantage of using GS over MAS is that it considers all markers irrespective of their role in phenotype. That is, it considers the entire marker irrespective of major or minor effects rather than targeting QTL-associated markers having major effect on the trait (Bernardo 2008). Another major advantage of GS is that the extent

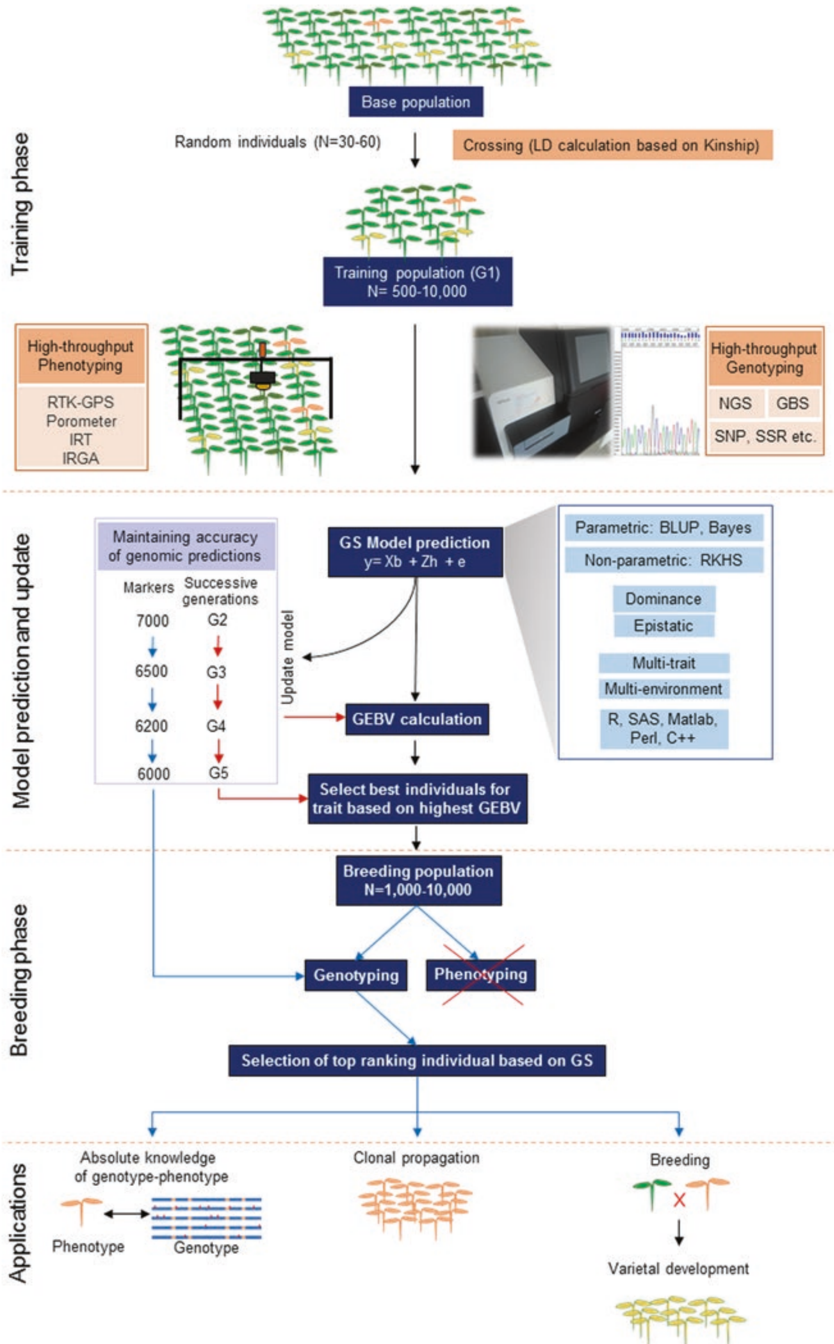

Fig. 3.2 Graphical representation of genomic selection approaches for the selection of elite line and the development of accurate GS model to obtain precise breeding value

of genetic gains achieved per year is several folds using genomic selection than traditional breeding or MAS approaches (Bernardo and Yu 2007; Heffner et al. 2010; Albrecht et al. 2011). Studies have shown that genotyping using genome-wide markers resulted in better predictions for selection of superior individual over a marker-assisted selection (Bernardo and Yu). High-throughput automated sequencing has significantly reduced the genotyping cost. GS is advantageous over conventional breeding as it circumvents phenotypic evaluation and QTL mapping and thus reduces the time for enhancing genetic gains and overall cost (up to 50%) for selecting individuals for breeding and varietal development. GS has been successfully utilized for enhancing genetic gains in several cereals (such as wheat and maize) and legume (such as chickpea, groundnut, soybean) crops.

3.2.5 Genetic Gains

Basically the aim of genetic gain is to get future offspring of plants or animals that can produce in much better way under future challenges and circumstances. So genetic gain is the measurement parameter of genetic improvement, and this is measured by final yield difference between our selected population and its progeny. The term genetic gain can also be defined as better than previous ones that "Genetic gain is the increase in the amount of yield that is produced yearly through genomic selection." The estimated genetic gain for a year is described by Falconer and Mackay (). It can be calculated by the formula:

$$\Delta G = ir\sigma A / t$$

where:

ΔG = selection response
i = selection intensity
r = accuracy of selection
σA = standard deviation of breeding values

To meet the demands of mankind, concern about food and feed, and various industrial uses, the potential of genetic gain should increase continuously in a positive linear progress method. For example, three global major crops in the world maize, wheat, and rice should have increase at annual rate of 1.16% and 1.31% so that we can cope up with the challenge of world population that is increasing 2% yearly. There are various factors present that can affect genetic gain like genetic variation present in selected population, heritability of selected trait, intensity of selection, and accuracy of selection (Xu et al. 2017). Apart from these challenges, there are various other environmental challenges also present. Climatic changes due to global warming and others factors, sort of arable land, and water availability, these all are additional challenges for minimizing the yield gap (Brummer et al. 2011).

There are remarkable examples present in animal science like dairy industries in the context of GS (Garner et al. 2016; Lu et al. 2016). In crop studies, impressing work has been done to increase the yield of maize through GS. A global and famous disease called stem rust has resulted in huge wheat yield loss in various countries. With the help of genomic selection, stem rust-resistant wheat was developed to increase the yield in wheat (Rutkoski et al. 2015).

Therefore, to meet our future demand, further improvements are required in the field of genetic gain, like good molecular breeding practices, agronomic practices, and utilization of newly molecular practices like CRISPR and better understanding of genomic traits. Another factor which also considered is proper communication between public and breeders so that new varieties can efficiently reach to the farmers.

3.2.6 Genetic Estimation and Prediction

Genomic selection has gained popularity by eliminating the need of various rounds of phenotypic selection by use of marker data and thus enhancing the actual rate of genetic gain by limiting the time and cost (Desta and Ortiz 2014). The prediction of the complex traits and identification of significant markers considering the whole genome and then combining them with the phenotype is becoming important in plant breeding to estimate the accurate GEBVs (Bernardo). To improve this accuracy, some of the earliest attempts involve combining the markers in a multiple regression model (Lande and Thompson 1990). Various parametric and nonparametric statistical methods have been evolved from years, and one of the prime method which was proposed for GS was ridge regression (RR) equivalent to BLUP in frame of reference to mixed models (Meuwissen et al. 2001). In comparison to ordinary regression in which the marker number cannot exceed the observation number, RR do not have such limitation. Other set of models for GS in plant breeding programs include models such as weighted Bayesian shrinkage regression (wBSR, BayesB fast version) and random forest (RF) (a machine learning method capturing the non-additive effects) and the Bayesian LASSO. However, linear models like GBLUP and machine learning algorithm have been predicted to be more successful in making the correct decision by recognizing the complex traits (Crossa et al. 2017). Perez-Rodriguez et al. (2012) showed a consistent superiority of RKHS and RBFNN over the Bayesian LASSO, Bayesian ridge regression, BayesA, and BayesB models in a study conducted on wheat for the days to heading and yield of grain. However, Roorkiwal et al. (2016) demonstrated RR-BLUP, BayesCπ, BayesB, Bayesian LASSO, and RF results with the same accuracy for traits of interest while studying chickpea.

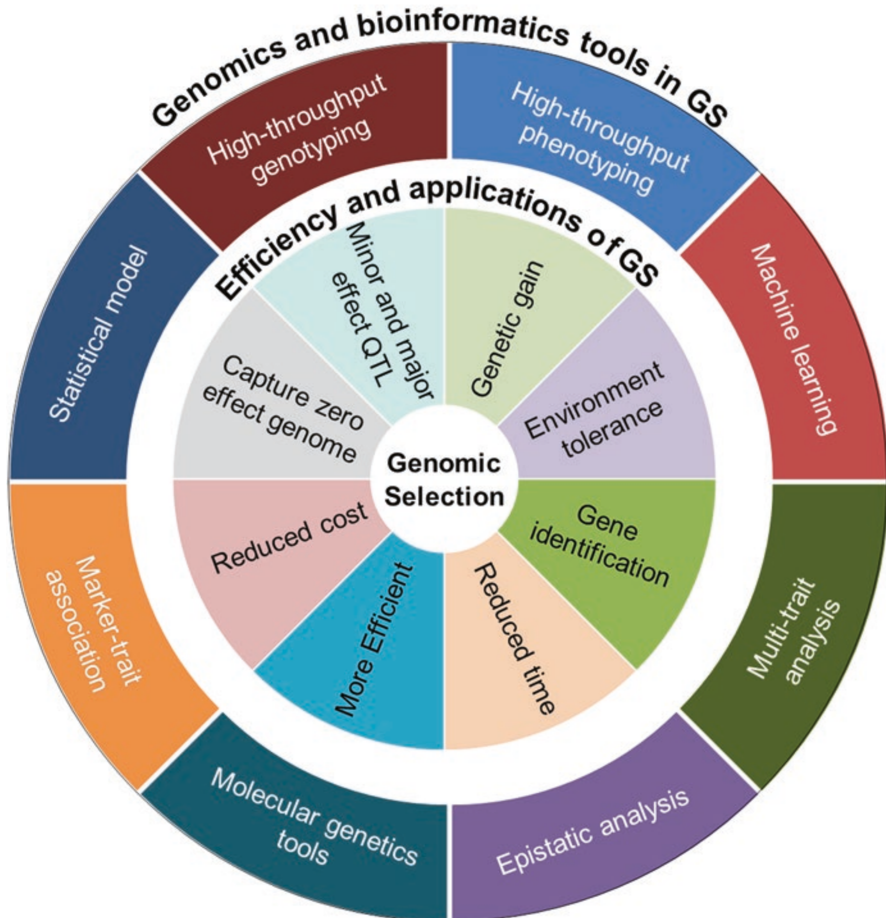

Fig. 3.3 Present studies and assessing of GS in diverse aspects of crop improvement

3.2.7 Integration of Bioinformatics and Genomics Tools in GS

With the advancements in NGS technologies, large-scale genome/transcriptome sequencing in different plant species resulted in generation of vast genomic resources. Since few decades, a variety of marker approaches have been developed and extensively utilized for establishing linkage between markers and genes, discovering QTL, pyramiding desired genes, and performing marker-assisted selection for combining of desired traits (Fig. 3.3). Tremendous advancement in the DNA sequencing technologies, including the advent of various next-generation sequencing (NGS) platforms in the last decade, transformed the conventional plant breeding to "next-generation plant breeding" (Schnable 2013). In the past, different type of marker system has been used, but in the era of next-generation sequencing, SNPs

have replaced all the techniques; SNPs are the smallest unit of polymorphism and provide the infinite source of markers for any individual. Large-scale genotyping with SNPs is a routine process in current projects which focus on all major crop species including cereals (Rasheed et al. 2017).

Markers which are very tightly linked to specific traits are diligently used to substitute the phenotypic variance. Multiple marker sets have to be analyzed in larger panels for MAS and analysis of genetic relationships and in the genetic mapping of different important traits. High-throughput genotyping is the method of multiplexed SNP markers that have revolutionized the system with reduced cost (Ganal et al. 2019), and highly automated pipelines are readily available to analyze thousands of sample in a day in large breeding programs. Due to very large genome size of cereals, in the past, markers have been discerned mainly based on comparative sequencing of amplicon, transcriptome analysis, and sequencing of some parts of the genome which leads to identification of only 1000–10,000 markers (Ganal et al. 2009), but these markers give some biased type of results which revealed that polymorphism is skewed toward analyzed samples. Recently sequence capturing is the technique of choice which targets the exon or coding part of genome and successfully reported in wheat and barley (Winfield et al. 2012). With the advancement of NGS, another approach is GBS, mainly used for SNP identification based on assemble sequence reads in contrast to readily available reference sequence information or alternative parent and analysis of allelic state (homozygous allele 1, heterozygous, homozygous allele 2).

Particularly, GBS is widely used in two different variations: the first is low redundancy resequencing of genomes (0.5–2× genome coverage) for the discerning of SNPs and also for scoring individuals of a mapping population or varieties (Lam et al. 2010), and the second one is complexity reduction to reduce the size of genome from being sequenced (Elshire et al. 2011). Many key crops have been studied by using GBS such as chickpea (Kujur et al. 2015), maize (Elshire et al. 2011), sorghum (Morris et al. 2013), and wheat (Poland et al. 2012). Third approach is array-based genotyping that detect certain number of SNP markers by using specific flanking sequences for assayed SNPs and define the respective loci by their sequence. These oligos are fixed to a chip, and calling of SNPs is done by using allele-distinctive fluorophores in the allele-specific detection process. Recently these array-based techniques have been developed and used for different cereals crops like wheat and rice.

On the other side, whenever there is requirement that the analysis of many samples has to be performed with few markers in a cost-efficient way (Thomson 2014), KASP (Kompetitive Allele-Specific PCR) assay technology has to be used. It does not require fluorescently labeled primer which is the main benefit of KASP. Two distinctively tailed unlabeled allele-specific forward primers are used for allele-specific amplification in combination with a single reverse primer. Due to this general allele calling scheme, the KASP marker system has very low assay costs compared with the TaqMan system. The KASP platform is commonly used in plant breeding projects. These days, high-throughput genotyping is a valuable tool in the research of cereal breeding. This includes applications in MAS of traits of interest,

germplasm characterization, and genetic relationship analysis and also has applicability in GWAS and genomic selection.

These days, there is novel concept of integration of genomic selection with speed breeding as a robust system to increase the yield of spring wheat. In this approach, they use simulations to unravel the potential of genetic gains by following a combined approach on real data set and breeding schemes. This leads to the selection among candidate genotypes directly after being generated, rather than after years of extensive phenotyping. This will help breeders to shorten the duration of the breeding cycle and consecutively results in genetic gain per unit of time (Lorenzana and Bernardo 2009).

3.3 Prediction and Evaluation of Breeding Scheme

Prediction of accuracy of various breeding schemes in genomic selection rely on a number of factors such as training populations and breeding populations and the relationship between the two when utilized within the program, genetic architecture of populations, and effect of environmental factors on breeding. Breeding schemes for genomic selection are evaluated on the basis of phenotypes or marker genotypes.

3.3.1 Breeding Schemes

Various breeding schemes for genomic selection can be divided into categories wherein phenotypic selection is conducted and where it is not. Different schemes are however identical in the fact that they aim to decrease time of breeding. As described by Bassi et al. (2016), breeding schemes when implemented in crops such as wheat have been categorized into different groups.

3.3.1.1 F_2 Recurrent Mass Genomic Selection

The scheme aims at reducing time of each breeding cycle. The breeding scheme is advantageous as most of the steps can be carried out in a greenhouse facility. This breeding scheme does not utilize phenotypic selection as opposed to other schemes. Initially, F2 plants are obtained from bi-parental populations which have been generated as a result of artificial crossing among desired parental lines. Genotyped markers known to impact traits of interest are used to identify genes and genetic diversities of population, and subsequently GEBVs are predicted. Individuals with high GEBV and genetic diversity are selected to generate the training population. Training populations are obtained from double haploids grown as F3. Further in the next cycle, F2 sub-population individuals obtained from initial populations as well as double haploids are genotyped. Following selection procedure from previous

cycle, individuals with high GEBV are further selected. Sub-sub-populations derived from the previous sub-populations are now grown in the next growing season and genotyped and selected on the basis of maximum GEBV. Initial yield trials for double haploid training populations can now be conducted, and GEBV is also calculated. Finally, a recombination cycle is feasible due to high speed of the breeding scheme. In the final season, sub-sub-populations of F2 individuals are grown and genotyped and undergo genomic selection. On the basis of GEBV calculated from double haploid- 3 (DH-3) training population, plants are selected from sub-sub-sub-populations for further normal breeding phase.

3.3.1.2 F3 Recurrent Genomic Selection

Where F2 recurrent genomic selection does not incorporate phenotypic selection, the present breeding scheme includes phenotypic selection at F2 generation. Phenotypic selection is used to associate markers with desired superior phenotypes, which as a result leads to reduction in the number of individuals which have to be genotyped (Wang et al.; Xu et al. 2018). In addition to growing generations within a greenhouse, F3 recurrent genomic selection grows populations on field also. In the initial cycle, bi-parental populations are generated and grown in field to visualize phenotypic characteristics, which are then used to select and reject 25% of crosses, termed as "among"-population selection. Further "within"-population selection is used with 2.5% selection intensity on the remainder of individuals obtained after "among"-population selection. Subsequent genotyping of F2 selected individuals grown under artificial environment is done. Marker-assisted selection is then used to identify genes responsible for important traits, which are also then used to deduce genetic diversity between populations. Calculation of GEBV is now conducted from the training population. Pairs of full-sibs are then selected for artificial crossing based on genes selected from marker-assisted selection, maximum genetic diversity, and high values of GEBV. The artificial crossing is used to generate subsequent sub-populations. Full-sib training populations are obtained from F3 populations. Single seed descent is employed on F4 individuals for further breeding. Sub-populations are again grown in field and visualized for phenotypes to select better populations, whereas the rest are discarded. Also, preliminary yield trait data is obtained from F4:5 training populations. Subsequently, F6 generations are obtained from training populations. Further, selected F2 individuals are grown in greenhouse. Moreover, F6 individuals from different training populations form the plants for training population, which are further genotyped. Preliminary yield traits are then used to calculate GEBV, which are used for selection of sub-populations from initial populations. The following cycle comprises of F2 individuals of sub-sub-populations which are phenotypically selected after being grown in field. Twenty-five percent sub-sub-populations are rejected on the basis of phenotypic selection, and F2 selected individuals are identified. Also F3 seeds are obtained for further breeding steps. In addition, F6:8 selected training population individuals are

utilized to perform advanced yield trials. Quality and disease-related traits are also observed during different on- and off-season. F9 seeds of training population and F3 sub-sub-population individuals are produced and grown in greenhouse and consequently genotyped. This data is utilized for calculation of GEBV, and individuals with low scores are discarded. High-scoring sub-sub-populations individuals are mated and utilized for normal breeding selection.

3.3.1.3 F4 Recurrent Genomic Selection

The F4 recurrent genomic selection scheme utilized genomic selection at F2 and F3 generations. This scheme is advantageous to previous F3 recurrent genomic selection which is phenotypically selected at F2 generation in quicker generation of full-sib training population as well as better precision of population selection. Better prediction of GEBV is accounted for due to calculation at F7 generation of training population and preliminary yield traits at F5:6. In addition, GEBV calculation is conducted for different time periods across a number of years and varying locations for F7:8 and F7:9 populations.

3.3.1.4 F7 Recurrent Genomic Selection

This breeding scheme entails phenotypic selection at F6 generation. Preceding steps until initial preliminary yield trials are concurrent with aforementioned breeding scheme. In addition, this method includes genotyping of F6 populations under field trials as well. Individuals with maximum GEBV are selected for further crossing. The scheme varies predominantly due to time taken for generation of double haploid seeds in sufficient amounts, so that preliminary yield trials are successfully conducted.

While following different breeding schemes for genomic selection, it is observed that training population closely related to breeding population results in better selection accuracy as compared to when the two are distantly related or completely unrelated. Comparison of total gain among different breeding schemes has shown that F2 recurrent breeding shows the best results. Although F3 and F4 lead to better genetic gains as compared to classical breeding, they are relatively similar among themselves.

3.3.2 Marker-Assisted Selection (MAS)

Markers are genetic regions located closely to genes, which represent variations between organisms. Marker-assisted selection (MAS) or marker-assisted breeding (MAB) or marker-aided selection (MAS) utilizes the presence or absence of genetic

markers which are linked to ergonomically important traits as molecular tools to aid efficient phenotypic selection. Common genetic markers include simple sequence repeats (SSRs), single nucleotide polymorphisms (SNPs), sequence tagged sites (STS), restriction fragment length polymorphism (RFLP), etc. Marker-assisted selection follows the steps of population development, quantitative trait loci (QTL) mapping, QTL validation, and marker validation. Recombinant inbred lines (RILs) or double haploid populations which can be stably maintained for repeated experiments are selected for MAS. Further, QTL mapping is performed due to the genetic linkage between markers and genes/QTLs of interest. QTL validation allows verification of effects of QTLs and genes, whereas marker validation helps in confirmation of specific genetic regions to be associated with particular traits (Collard and Mackill 2007). MAS allows identification of individuals with desired QTLs and genes according to genetic markers linked to them and hence allows faster selection for downstream analysis.

MAS is pivotal in gene pyramiding wherein a number of important genes for a trait are assembled in a unique genotype. It has also been used for breeding of biotic and abiotic stress-resistant crops and for improvement in grain quality-related traits like protein content in *Zea mays* and fatty acids in *Glycine max*. MAS have also been successfully utilized for transfer of genes between cultivars. In addition, it has been proven to be effective for genetic introgression from wild to cultivated varieties. Marker-assisted backcrossing is one such application of MAS. It is essential to use MAS when phenotyping of traits is expensive or not feasible or when trait heritability is low. MAS have also proved useful for recessive genes controlling important agronomic traits when co-dominant markers are used. Moreover, it is independent of environmental effects and therefore allows selection under different conditions.

In addition to several advantages, MAS has some limitations as well. Markers with limited polymorphisms are not amenable for MAS. Also, MAS is advantageous primarily for monogenic or oligogenic traits.

3.3.2.1 Models for Marker Effect

Various models for estimating effect of markers in genomic selection have been devised. Some of these methods are least square (LS), best linear unbiased prediction (BLUP), and Bayesian (Bayes) estimation and machine learning method. LS, BLUP, and Bayes' method and its variants are categorized under parametric methods, whereas machine learning methods are included under non-parametric heading. Since the number of markers often exceeds the number of individuals, it is difficult to study the effect of all markers simultaneously due to limited number of freedoms. Methods such as BLUP, Bayes estimation, and machine learning address this problem by considering marker effects as random.

3.3.2.2 Least Square (LS) Method

The method checks individually statistical significance for all genes. While estimating effects, genes with non-significant effect take values equal to zero. Due to limited number of degrees of freedom, all marker effects cannot be estimated simultaneously (Meuwissen 2003).

3.3.2.3 Best Linear Unbiased Prediction (BLUP) Method

BLUP is utilized for estimation of random effects in linear mixed models where both fixed and random effects are accounted for. In the case of BLUP, marker effects can be estimated simultaneously since the degrees of freedom are not required for random effects. BLUP model is represented as

$$y = X\beta + Zu + e$$

where:

y = vector for phenotypes
X and Z = known matrices
β = signifies fixed effects which are non-genetic
u = vector for random regression coefficients
e = residual vector

BLUP includes information related to pedigrees, full-sibs, and half-sibs.

3.3.2.4 Bayesian Estimation

In the case of Bayesian estimation, variance of marker effects is different for various genes and is deduced by using a prior variance, given by

$$V = 0 \quad \text{for probability } p$$

$$V \sim \chi^{-2}(v, S) \quad \text{for probability } (1 - p)$$

where:

v = degrees of freedom
S = scaling parameter
p = varies with genetic mutation rate
Also, v and S change with mutation effect distribution.

Markov chain Monte Carlo (MCMC) is utilized to deduce model parameters in Bayesian methods.

3.3.2.5 Machine Learning

In machine learning non-parametric methods, support vector machine (SVM), random forest (RF), and radial basis function neural network (RBFNN) are also being used for prediction of marker effects. Although other methods are recently being used, SVM remains the most widely used machine learning method. SVM are effective for classification and regression analysis and include combinations of unsupervised learning methods. SVM considers complexity of data under training population along with fitting of samples. To compensate for computational complexity which arises as a result of large input data set, various kernel functions are utilized which solves dimensionality problem and also considers various features of training samples. One of the most widely used kernels is Gauss RBF kernel.

3.3.2.6 Prediction of Total Genetic Value: High-Throughput Genotyping (SNPs)

Single nucleotide polymorphic markers are the most abundant form of markers which are genotyped and used in linkage analysis. Various methods for SNP genotyping are used such as sequencing, restriction fragment length polymorphism (RFLP), and amplification refractory mutation system (ARMS), among others. High-throughput genotyping techniques have enabled generation of high-density maps for molecular markers. Genetic value or genomic estimated breeding value (GEBV) utilizes high-density markers such as SNPs and is based on major marker as well as minor marker effects. This enables inference of better genetic variation for a trait of interest. GEBV enables selection of individuals in breeding as compared to marker genotypes as used in MAS.

Various models have been used for prediction of genetic value/GEBV such as least square (LS) estimation, BLUP, and Bayesian method variants (BayesA, BayesB, BayesC, BayesR, BayesCpi). LS method involves handling data with insufficient degrees of freedom. BLUP enables analysis of unbalanced data as well as relationship information. BLUP is unbiased because the mean estimate value is equal to the average value of trait. Prediction accuracies of Bayes' method have been seen to be better than GBLUP (genomic best linear unbiased prediction) based on several simulation studies (Meuwissen 2003). GEBV prediction accuracies also depend on several factors such as:

- Linkage disequilibrium
- Population size
- Population structure
- Marker density
- Pedigree information or kinship

3.4 Statistical Model of GS

To improve the accuracy of genomic predictions, several statistical models have been employed. The common aim of the entire model is to correlate variation in marker loci to observed phenotype and to minimize the deviation from true genetic values. Models are classified as parametric and non-parametric. The popular parametric models include linear least square regression, best linear unbiased prediction (BLUP), and the Bayesian model, while the non-parametric model includes reproducing kernel Hilbert space (RKHS) regression (De los Campos 2012). Some of the popular models are discussed below:

3.4.1 Least Square

LS is the basic model to predict breeding value of an individual based on genotype x phenotype relationships. This model tests all genes one by one for their statistical significance and sets the effect on non-significant genes to zero. Readers may refer to Howard et al. (2014) for further details.

$$y_i = \mu + \sum_{p}^{j=1} X_{ij} m_j + e_i$$

where:

y_i = vector of phenotypes
μ = overall mean
X = incidence matrix
i = individual
j = marker
p = position of marker
m_j = random effect associated with marker j
e_i = random residual

Limitation One of the major limitations affecting the prediction is that the number of maker loci is significantly large compared to the number of observations (phenotype of individuals) to estimate the effect of all marker at the same time. Least square models become invalid when effects of all the markers are estimated, simultaneously. To overcome the limitation, other models were developed.

3.4.2 BLUP and BLUE

BLUP (best linear unbiased prediction) model is one of the most popular models for genetic predictions (Henderson 1975). This model is based upon the linear mixed model that incorporates genomic relationship matrix. The model can accommodate pedigree information (kinship data) for defining the covariance among familiar relatives (Bernardo 2010). BLUP assumes that all genes have small but non-zero effect on the trait. BLUP fits all the allelic effects as random effects (considering variance-covariance) that are calculated simultaneously. Fixed effects are estimated using BLUE (best linear unbiased estimation). Such fixed effects are constant variables rather than random ones and do not have variances and co-variances. For detailed information about BLUP model and prediction, readers may refer to Robinson (1991), Clark and van der Werf (2013), and Howard et al. (2014).

The mixed effect model is denoted by the following equation:

$$y = X\beta + Z\alpha + \varepsilon$$

where:

Y = vector of phenotypes
X = incidence matrix of fixed effects
β = vector of fixed effects
Z = incidence matrix for random effects
α = vector of random effects
ε = vector of residual error

3.4.3 Bayesian Framework

Bayesian model is similar to BLUP except that it allows some markers to have zero effect and others to have low to moderate effects. The model is computationally more demanding but provides more accurate estimates. It considers different variance of allelic effects for each gene. Bayesian A uses inverted chi-square to regress the marker variance to zero and assumes that all marker effects be >0. Bayesian B presumes that not all markers contribute to the genetic variation. Improved Bayesian methods such as Bayesian C and Bayesian π were developed by Habier et al. (2011) to deal with the weakness of Bayesian A and Bayesian B. However, for the traits affected by dominance and genotype x environment interactions, non-parametric models such as reproducing kernel Hilbert space (RKHS) would yield more accurate estimations (Howard et al. 2014).

3.4.4 *Performance of Statistical Model in GS*

Studies have shown that the parametric method such as BLUP performs well for most traits and BayesB yields slightly higher predictive accuracy for traits with large-effect QTLs (De los Campos 2012). Accuracy of predications was very high in Bayesian model followed by BLUP and even less in least square model (Meuwissen et al. 2001). RKHS model yielded prediction accuracies 42–48% for multiple diseases in wheat, while the authors found that, for the two extensive wheat data sets, the RKHS/GK gave prediction accuracies up to 17% higher than GBLUP linear model (Gianola and van Kaam 2008). Non-parametric methods perform better than parametric methods when an epistatic effect exists in a population (Howard et al. 2014). From simulated data, the Bayesian approach was shown to have better performance in prediction accuracy than linear LS regression, RR, and BLUP (Meuwissen et al. 2001; Habier et al. 2009, 2010).

3.4.4.1 Factors Influencing the Accuracy of Genomic Prediction

The accuracy of the prediction relies on several factors such as size of training population, heritability of trait, relatedness of training population with breeding population, type of genetic marker, G × E interaction, number of genome-wide markers, epistatic effect, and type of statistical model (Lorenz et al. 2011; Pszczola 2012; Howard et al. 2014; Arruda 2015). Simulation studies have shown that genetic architecture is the most crucial factor governing the accuracy of prediction (Daetwyler et al. 2010; Wimmer et al. 2013). The size of training population should be sufficiently large and must be related (half-/full-sib) to the breeding population (Isidro et al. 2015). Estimates suggest that at least 50, 100, and 1000 should be the size of full-sib, half-sib, and less related training population for getting accuracies above 0.5. Further, traits showing lower heritability need even larger training set. When the phenotype is the effect of epistasis, parametric model is unable to predict the genetic values accurately. For phenotypes in which epistatic effect plays major role, non-parametric models are better suited for predictions (Gianola et al. 2006).

3.5 Statistical Concept of GS

All the methods of selection, simple phenotypic, marker-assisted, and genomic are based on finding an association between underlying genetic factors and trait of interest (Nakaya and Isobe 2012). To comprehend the concept of GS, first we need to understand the genetic models employed for phenotyping and then decompose the variance component until it is explained by DNA markers.

As widely known, only genetic factors are hereditary in nature which forms the basis of any genetic model for analysis of quantitative traits. However, the phenotype is the combination of both genotype and environment.

$$\mathrm{P} = \mathrm{G} + \mathrm{E}$$

where:

P = phenotypic mean
G = genotypic effect
E = residual environmental effect

Here, the genotypic effect includes additive, dominance, and epistatic components (Fisher 1918). Therefore, the phenotypic variance will be expressed as follows:

$$V(\mathrm{P}) = V(\mathrm{G}) + V(\mathrm{E}) + 2\mathrm{Cov}(\mathrm{G,E}) = V(\mathrm{G}) + V(\mathrm{E})$$

where:

V(P), V(G), and V(E) = phenotypic, genotypic, and environmental variance
Cov(G, E) = covariance between genotype and environment

However, in the above model, the interaction between genotype × environments (G × E effect) is assumed to be zero.

Furthermore, heritability is the proportion of total phenotypic variance accounted by genotype. It is of two types: broad (H^2) and narrow sense (h^2). The broad sense heritability includes the total genetic component, viz., additive, dominance, and epistatic effects, while narrow sense heritability (h^2) only accounts for additive genetic effect which is truly heritable in nature and responds to selection. Thus, the genetic model (P = G + E) can be rewritten using additive genetic component (A) as

$$\mathrm{P} = \mathrm{A} + \mathrm{E}'$$

where:

E′ = residual effects which are not involved in the additive genetic effect. So, E′ also contains dominant and epistatic components.

The variance for the above equation can be rewritten as

$$V(\mathrm{P}) = V(\mathrm{A}) + V(\mathrm{E}')$$

Moreover, h^2 by definition is the ratio between V(A) to V(P):

$$h^2 = V(\mathrm{A}) / V(\mathrm{P})$$

Therefore, in GS, the additive component *V*(A) is further broken down into variances explained by several DNA markers, $V(A_1)$, $V(A_2)$, …, $V(A_n)$, with an assumption that these markers do not interact among each other (Meuwissen et al. 2001).

3.6 Efficacy and Power of GS

The prediction efficacy of GEBV can be defined as Pearson correlation between GEBV and experimentally estimated breeding value (EBV), *r*(GEBV:EBV); the EBV value can be assessed using phenotypic mean values. Thus, the degree of correlation offers estimate of selection accuracy which is directly proportional to selection response based on GEBV (Falconer and Mackay 1996). Although depending upon heritability and genetic constitution of traits, phenotypic selection sometimes performs better than GS with regard to gain per cycle. However, with respect to genetic gain per unit time, it lags behind as the time required for one cycle of GS is one-third than that of phenotypic selection for numerous crop species (Lorenzana and Bernardo 2009; Heffner et al. 2010). The gain per unit time is even more pronounced in those species which are perennial in nature and require several seasons of estimation due to longer breeding cycle, viz., oil palm (Wong and Bernardo 2008). Still most of the simulation and empirical studies have continuously showed superiority of GS in terms of genetic gain per cycle and accuracy over traditional MAS and phenotypic selection based on pedigree information alone (Bernardo and Yu 2007; Singh and Singh 2015; Crossa et al. 2017; Yabe et al. 2018; Guo et al. 2019; Hu et al. 2019). A comparative feature of GS over MAS is listed in Table 3.1. The accuracy of GEBV chiefly depends on factors (Table 3.2) such as effective population size, marker type and density, heritability of trait, and kinship. However, recent effort to optimize GS using genetic algorithm on TNP was proposed by Akdemir et al. (2015) for *Arabidopsis*, wheat, rice and maize which showed improved accuracies when the same set of individuals were subjected to their model. Thus GS is the profitable strategy in breeding program. Along with this breeding goal, we can perform GS for low heritability of complex trait using genomic information that is moderately explained by association mapping. The integration of

Table 3.1 Summary of genomic selection features over marker-assisted selection

Features	Marker-assisted selection	Genomic selection
Target QTLs	QTL with major effects	QTL with small effects
Phenotype and genotype scoring	Mapping population	A subset of training population
Objective of training phase	Identification of markers linked to QTLs	Models developed for GEBV
Objective of breeding phase	Markers linked to target QTL used	Genome-wide genotype data considered
Selection of lines	Individual with marker genotype	GEBVs used for selection
Genetic variance of lines	Individual marker effect	All marker effects of GEBVs

Table 3.2 List of genomic selection studies in various plant species

Crop	Population type[a]	Training population size	Marker[b]	Prediction model[c]	Traits	Accuracy	References
Soybean	Genotypes	363	84,416 SNPs	RR-BLUP	Soybean cyst nematode resistance	0.41–0.52	Wen et al. (2019)
Soybean	RILs	540	2647 SNPs	RR-BLUP	Protein, oil, yield	0.81, 0.71, 0.26	Stewart-Brown et al. (2019)
Wheat	DHs	282	7426 SNPs	RKHS, RF	Grain yield, kernel weight, wheat protein content, sodium dodecyl sulfate (SDS) sedimentation volume	0.42–0.43, 0.26–0.44, 0.17–0.36, 0.52–0.70	Hu et al. (2019)
Rice	$F1_s$	1495	102,795 SNPs	BLUP	Ten agronomic traits	0.35–0.92	Cui et al. (2019)
Rice	NCII population	575	3kRG filtered and core SNP set	GBLUP, LASSO, BayesB, PLS, SVM, RKHS	Eight agronomic traits	0.37–0.86	Xu et al. (2018)
Banana	Genotypes	307	10,807 BA-SNPs	BRR, LASSO BayesA, B, and C RKHS	Fruit filling, fruit bunch	0.47–0.75	Nyine et al. (2018)
Wheat	Elite breeding population	273	~90,000 SNPs	RR-BLUP	Nine agronomic and biochemical traits	>0.25	Huang et al. (2016)
Tomato	$F1_s$	96	337 SNPs	GBLUP, Bayesian Lasso, wBSR, BayesC, RKHS, RF	Soluble solid content Total fruit weight	0.56–0.68, 0.22–0.27	Yamamoto et al. (2017)
Tomato	Genotypes	163	5995 SNPs	RR-BLUP	35 metabolic traits Ten quality traits	0.05–0.81	Duangjit et al. (2016)
Rice		369	73,147 SNPs	RR-BLUP	Grain yield, plant height, flowering time	0.31, 0.34, 0.63	Spindel and Iwata (2018)

Rye	Test cross	828	14,269 SNPs	RR-BLUP, random forest (RF)	Grain dry yield Plant height Thousand kernel weight	0.19–0.48 0.47–0.80 0.44–0.74	Durán (2015)
Apple	20 full-sib families	977	7829 SNPs	BLUP, BayesC_π	Attractiveness Fruit cropping Fruit size	0.21 0.08 0.23	Muranty et al. (2015)
Rice	Inbred lines	413	36,901 SNPs		Protein content, deed width	0.44, 0.84	Guo et al. (2014)
Maize	Inbred lines	257	48,814 SNPs	GBLUP	Protein content	0.29	Guo et al. (2014)
Maize	Individuals from 11 families	930	152–210 SNPs	RR-BLUP	Grain yield Grain moisture	0.5–0.6 0.4–0.8	Zhao et al. (2012a, b)
Rice	Inbred lines	210	270,820 SNPs	LASSO	Yield Apiculus colour	0.16 0.98	Xu (2013)
Sugar beet	Inbred lines	924	677 SNPs	RR-BLUP	Nitrogen content White sugar yield	~0.5 ~0.9	Wurschum et al. (2013)
Arabidopsis thaliana	Inbred lines	199	215,908 SNPs	RR-BLUP	Plant width Flowering time	~0.45 0.65–0.75	Wimmer et al. (2013)
Wheat	Lines CIMMYT	254	2056 SNPs	RR-BLUP	Yield Date to heading	0.43–0.51 0.60–0.66	Wimmer et al. (2013)
Rice	Varieties from 6 sub-populations	413	36,901 SNPs	LASSO, the elastic net, BayesB, RR-BLUP	Flowering time	0.46–0.59	Wimmer et al. (2013)
Maize	Inbred lines	197	37,908 SNPs	GBLUP	Northern corn Leaf blight	~0.7	Technow et al. (2013)

(continued)

Table 3.2 (continued)

Crop	Population type[a]	Training population size	Marker[b]	Prediction model[c]	Traits	Accuracy	References
Maize	DHs	635	16,741 SNPs	GBLUP	Kernels per row	0.3–0.6	Riedelsheimer and Melchinger (2013)
Alfalfa	Individuals from one breeding population	384	~10,000 SNPs	RR-BLUP	Biomass yield	0.21–0.66	Li et al. (2015)
Barley	DHs	996	335 SNPs	Bayes-LASSO	Grain yield	~0.6	Heslot et al. (2013)
Wheat	Lines bred in CIMMYT	622	34,843 SNPs	GBLUP	Grain yield	~0.2	Dawson et al. (2013)
Wheat	Watkins landraces	222	5568 SNPs	GBLUP, Bayes-R	Stem rust Stripe rust	0.2–0.4 0.2–0.4	Daetwyler et al. (2013)
Maize	Lines from half-diallel cross 4 inbred	788	272–469 SNPs	GBLUP	Grain yield	~0.6	Zhao et al. (2012a)
Maize	Inbred lines	255	37403 SNPs	RR-BLUP	Grain yield, anthesis-silking interval	~0.5, ~0.5	Windhausen et al. (2012)
Wheat	Lines from 15 public and three private breeding	322	2402 DArTs, 8 SSRs	RR-BLUP, Bayes-LASSO, RKHS, RF	Heading date	~0.3	Rutkoski et al. (2012)
Maize	Test cross lines	570	38,091 SNPs	RR-BLUP	Sugar content	0.69	Riedelsheimer et al. (2012)
Loblolly pine	Individuals from 32 parents	951	4853 SNPs	RR-BLUP, BayesA, CQ, LASSO	Lignin	0.17	Resende et al. (2012a)

Loblolly pine	Individuals from 32 parents	926	4825 SNPs	RR-BLUP	Diameter Breast Height Total height	0.65–0.75 0.65–0.75	Resende et al. (2012a)
Eucalyptus	Two elite breeding populations	738 and 920	3300 DArTs	RR-BLUP	Pulp yield	0.55	Resende et al. (2012b)
Wheat	Lines bred in CIMMYT	254	1726 DArTs 34,749 SNPs	GBLUP	Grain yield 1000 kernel weight	0.2–0.4~0.3	Poland et al. (2012)
Wheat	Lines bred in CIMMYT	306	1717 DArTs	RR-BLUP, BayesA, B, LASSO, RKHS, RBFNN, BRNN	Date to heading	0.5–0.6	Perez-Rodriguez et al. (2012)
Barley	$F1_s$	691	3072 SNPs	RR-BLUP, Bayes-CQ, LASSO	DeoxynivalenolFusarium head blight	0.5–0.7 0.5–0.7	Lorenz et al. (2011)
Apple	$F1_s$	1120	2500 SNPs	RR-BLUP, Bayes-LASSO	Astringency	0.68	Kumar et al. (2012)
Maize	NAM	(126–196) × 25	1106 SNPs	RR-BLUP	Three flowering traits	0.26–0.57	Guo et al. (2012)
Maize	Inbred lines	300	55,000 SNPs	Bayes-LASSO, RBFNN, RKHS	Gray leaf spot	0.2–0.5	Gonzalez-Camacho et al. (2012)
Cassava	Research accessions	358	390 SNPs	RR-BLUP	Dry matter content	0.67	de Oliveira et al. (2012)
Wheat	Lines bred in CIMMYT	599	1279 DArTs	Bayes-LASSO	Grain yield	0.5–0.6	Burgueno et al. ()

(continued)

Table 3.2 (continued)

Crop	Population type[a]	Training population size	Marker[b]	Prediction model[c]	Traits	Accuracy	References
Wheat	DHs	174	574 DArTs	RR-BLUP	Eight grain quality	0.41–0.73	
Wheat	DHs	209	399 SSRs, DArTs, AFLPs, TRAP, STS	RR-BLUP	Eight grain quality	0.32–0.84	Heffner et al. (2011)
Maize	Lines bred in CIMMYT	300	1152 SNPs	RR-BLUP	Northern corn leaf blight Gray leaf spot	~0.5 ~0.5	Crossa et al. (2011)
Wheat	Lines bred in CIMMYT	94	234 DArTs	Bayes-LASSO, RKHS	Grain yield	0.43–0.79	Crossa et al. (2011)
Maize	Lines bred in CIMMYT	300	1148 SNPs	M-BL	Grain yield, female flowering, male flowering, anthesis-silking interval	0.42–0.79	Crossa et al. (2010)
Wheat	Lines bred in CIMMYT	599	1279 DArTs	PM-RKHS	Grain yield	0.48–0.61	Crossa et al. (2010)
Maize	DHs	208	136 SNPs and SSRs	RR, POW, EXP, GAU, SPH	Kernel dry weight	1.00	Piepho (2009)
Barley	DHs	140	107 RFLPs and AFLPs	BLUP	Plant height, two chemical components	0.66–0.85	Lorenzana and Bernardo (2009)
Barley	DHs	150	223 RFLPs	BLUP	Grain yield, amylase activity	0.64, 0.83	Lorenzana and Bernardo (2009)
Maize	DHs	371	125 SNPs	BLUP	Three morphological traits, grain moisture	0.31–0.55	Lorenzana and Bernardo (2009)
Maize	$F2_s$	349	160 SSRs	BLUP	Three morphological traits, grain moisture	0.59–0.72	Lorenzana and Bernardo (2009)

Maize	RILs	119	1339 SSRs and RFLPs	BLUP	Five morphological traits, grain moisture	0.40–0.50	Lorenzana and Bernardo (2009)
Arabidopsis thaliana	RILs	415	69 SSRs	BLUP	Flowering time, dry matter, free amino acid	0.90–0.93	Lorenzana and Bernardo (2009)
Maize	RILs	223	1339 SSRs and RFLPs	BLUP	Eight morphological and three chemical traits, grain moisture	0.48–0.73	Lorenzana and Bernardo (2009)

[a]*RILs* recombinant inbred lines, *DHs* doubled haploids, *CIMMIYT* International Maize and Wheat Improvement Center, *NAM* nested association mapping, $F1_s$ first filial generation, $F2_s$ second filial generation, *NCII* North Carolina II design

[b]*SSR* simple sequence repeat, *RFLP* restriction fragment length polymorphism, *SNP* single nucleotide polymorphism, *AFLP* amplified fragment length polymorphism, *DArT* diversity arrays technology, *TRAP* target region amplification polymorphism, *STS* sequence-tagged site

[c]*BLUP* best linear unbiased prediction, *RR* ridge regression, *POW*, power; EXP, exponential, *GAU* Gaussian, *SPH* spherical, *RR-BLUP* ridge regression best linear unbiased prediction, *PM-RKHS* pedigree plus molecular marker model using reproducing kernel Hilbert space regression, *M-BL* Bayes LASSO, least absolute shrinkage and selection operator, *RKHS* reproducing kernel Hilbert space regression, *RBFNN* radial basis function neural networks, *GBLUP* genomic best linear unbiased prediction, *Bayes-R*, Bayes C, Bayes Cπ, *WBSR* weighted Bayesian shrinkage regression, *SVM* support vector machine, *PLS* partial least square, *RF* random forest

various genomics and bioinformatics tools into GS enhances the efficiency of GS, and the regular update of GS model at generation interval for the accuracy of model was found as the other benefit of GS in transition of traditional breeding scheme.

3.6.1 Effective Population Size

The concept of effective population size (*Ne*) can be defined as the number of randomly mating individuals giving rise to the observed rate of inbreeding in a population (Wright 1931, 1933). The size of TRP has a significant effect on accuracy as a linear trend has been observed between accuracy of GEBV prediction and training population (Van Raden et al. 2009). Therefore, an increase in size of TRP consequently improves prediction accuracy. Usually the necessity of higher ratio of TRP to BP is essential in situations when both the populations have larger genetic variability among each other, the breeding population is small in size, the character under study has lower heritability, and a greater number of QTLs are governing the character. Furthermore, self-pollinated species would require smaller TRP size than cross-pollinated species (Singh and Singh 2015). In a simulation study, the prediction accuracy of GEBV was found to be 0.848 when the TRP comprised of 2200 lines, but it reduced to 0.708 when the population size was decreased to 500 (Singh and Singh 2015). A 20% increase in accuracy was achieved when TRP size was increased (Lorenzana and Bernardo 2009).

3.6.2 Marker Type and Density

The type of marker also plays a crucial role in determining the number of markers required as shown in a study SNPs required was 2–3 times more than SSRs to achieve the same level of accuracy (Meuwissen 2009; Meuwissen et al. 2001). Also, marker density should be sufficient enough to capture maximum number of QTLs governing the character so as to obtain at least a single marker with strong LD associated with the QTL. Usually, the number of markers required is more for cross-pollinated than self-pollinated species. Furthermore, low heritability traits also require larger marker density (Singh and Singh 2015).

3.6.3 Heritability of Trait

Heritability is defined as the proportion of genetic variance which affects a phenotypic character. Usually as the value of heritability decreases, the prediction accuracy increases (Rutkoski et al. 2015; Singh and Singh 2015).

3.6.4 Kinship

Kinship can be defined as individuals sharing common ancestry. The TRN essentially should sufficiently capture the genetic diversity present in the BP for its appropriate representation. This can be attained by selecting those lines in TRN which share kinship with BP (Jannink et al. 2010). Simulation studies have revealed that when parental lines were excluded from TRN, the prediction accuracies decreased (Duran 2015; Duangjit et al. 2016; Mastrodomenico et al. 2019), indicating the importance of genetic relatedness.

3.7 Advantages and Disadvantages of GS

3.7.1 Advantages

Many empirical studies have revealed that the genetic gain and future demand of population in case of food can be achieved several fold with the help of genomic selection compared to traditional breeding programs. Primarily, by using MAS in breeding programs, we can target only few QTL s which have large effect on the trait, but GS uses genome-wide markers across the population to target both the QTLs having major and minor effects, based on predicted genomic estimated breeding value (GEBV) for much better and reliable selection. GS considers complex traits like yield which are governed by multiple genes (polygenic), G × E interaction, epistatic effects, and dominance. Genomic selection is advantageous in comparison to traditional breeding program because it reduces the cost of selecting individual for varietal development and breeding and also reduces the time for improving genetic gain. There is no need of phenotypic evaluation of BP in GS. Hence, it is very helpful in the selection of individuals for traits that cannot be phenotyped or appear late in their cycle like disease resistance or under some specific environmental conditions. In a study in maize, it has been shown that genomic selection is having larger effect compared to MAS, notably for all characters having low heritability (Shamshad and Sharma 2018).

3.7.2 Disadvantages

Regardless of the aforementioned advantages, GS has a few associated disadvantages too. Training population needs to be large enough to identify precise association between genotype and phenotype. It needs to be updated regularly by adding new genotypes because recombination or mutation during long time interval may cause the loss of previously estimated associated marker or QTLs to the phenotype. During successive generations, the predicted model requires to be updated. Precise

and high-throughput phenotyping are required to achieve the accurate and large amount of trait data that is required for commercial-scale GS. For the potential application of GS, there is a need to modify the statistical simulation so as to predict accurate environment-specific GEBV. In GS the species loses their ability to purge out the some unfavorable condition during selection such as inbreeding depression (McParland et al. 2009).

3.8 Perspectives

Primary motivation behind GS is to accomplish more extensive and reliable selection and to accelerate the genetic progress in plant breeding, so as to diminish the cost of time per cycle and reduce the generation interval by utilizing genome-wide markers (Wang et al. 2018). GS opens up a promising research direction for breeding science, and it has turned into a hot issue in recent quantitative genetics.

In recent time, high-throughput genotyping methods are being used to investigate the genome of any species and opening up new vistas of opportunities. Genome sequencing including metabolomic and transcriptomic data will provide important information for implementing genomic selection programs (Riedelsheimer et al.). Additional reduction in the cost of these sequencing methods will encourage wider adoption and implementation of new approaches for the benefit of crop research (Eggen 2012). GS advancement has improved the data complexity dramatically, by leading to integrate statistics, machine learning, genetics, and quantitative genetics, and bioinformatics sciences in interdisciplinary research. Various statistical methods have been implemented to predict non-phenotyped individuals in GS leading to greater overall GS efficiency. The performances of these methods depend on the genetic architecture of the specific trait. When using the same GS method, G × E interaction is an important factor to be considered like for the same trait in various environments, accuracies differ greatly because of interaction (Burgueno et al. 2012, Heslot et al. 2014). These GS prediction models will be helpful in different disciplines to develop genetically superior individuals by exploiting G × E interactions. Simultaneously, it is essential to focus on mining of large-scale crop databases to merge phenotype data from other resources and allow accessing unexplored diversity and crop improvement in breeding programs (Hassani-Pak and Rawlings 2017). Understanding of breeding program parameters like effective population size and marker density will assist to determine the trait heritability and extent of LD by analytical formulae. GS has been used to develop agronomically important individuals more quickly and enhance the rate of genetic gain in a few crops (Varshney 2016). Genetic gain can be further increased by increased population size and selection intensity. In case of hybrid breeding in self-pollinating crops like wheat, GS seems to be a propitious approach, especially when heterotic pools are not known (Zhao et al. 2015; Longin et al. 2014).

There are still some issues to be solved, to conquer the low level and changing pattern of LD in breeding populations as model updating is important for

maintaining the exactness of GS across selection cycles (Bernardo 2016). GS implementation in breeding sciences could be limited by two primary factors, first costs of genotyping and second unclear guidance as to where GS can be applied effectively in a breeding program. It would be necessary to develop strategies for potential improvement of genotyping cost-efficiency and a guideline for GS breeding and minimize cost without compromising the accuracy of predictions. Further empirical studies of genetic composition, effects of statistical models, TNP size, and different selection criteria and marker density are urgently needed for the effectiveness of GS.

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Chapter 4
Data-Driven Decisions for Accelerated Plant Breeding

Saritha V. Kuriakose, Ravindra Pushker, and Ebenezer M. Hyde

4.1 Introduction

Plant breeding is the method of developing improved crop varieties suited to the needs of farmers and consumers through the creation, selection, and fixation of beneficial phenotypic traits (Moose and Mumm 2008). Plant breeding was, at one time, as much art as labor-intensive science. The art of plant breeding like ancestral farming involved cross-pollinating parental plants with genetic potential to produce progenies with better yield or disease resistance. This genetic potential was often based on physical attributes of the plant. Breeders used their experience and the observed phenotypes of crops to choose offspring with better performance, so as to achieve genetic improvement of target traits (Wang et al. 2018). Though this process took decades to show results, plant breeding was enormously successful on a global scale as evidenced by the conversion of teosinte grass into corn, the development of hybrid maize (Duvick 2001), the introduction of wheat (*Triticum aestivum*) and rice (*Oryza sativa*) varieties that spawned green revolution (Evenson and Gollin 2003), and the recent commercialization of transgenic crops (James 2007). While the achievements are impressive, continued rapid population growth, environmental changes, and the associated need for a more sustainable, low-input agriculture necessitate accelerated innovation in plant breeding. In the current scenario of decreasing arable land, changing weather conditions, and alarming increase in crops lost to pest, the success of plant breeding is critical to meet planetary challenges of food and water security for the world's growing population.

Advances in plant breeding and management practices have contributed an annual gain of 0.8–1.2% in crop productivity (Li et al. 2018). Notwithstanding,

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plant breeders now need to develop varieties that can deter pests and diseases, maximize resource use efficiency, and have stable high yields despite changing climate, all while being sustainable. The linear growth in food production seen historically will not be enough to satisfy global demand by 2050, especially with water shortages and changes in agriculture landscape due to degradation and urbanization. As Wallace et al. pointed out "It is dangerous to assume that just because production has outpaced population growth in the past that it will inevitably do so in the future" (Wallace et al. 2018).

Although "green revolution" signaled the advent of modernization in farming, with the introduction of molecular breeding and genomic selection (GS), we are in an era of precision breeding where specific parts in the genome can be targeted to achieve individual breeding goals much faster. In addition to the techniques used in cross breeding, mutation breeding, and molecular breeding (detailed in sections below), recently developed targeted techniques like CRISPR/CAS system, NHEJ (non-homologous end joining), and HDR (homology directed repair pathway) are used in precision breeding to target specific plant traits such as drought or disease tolerance by editing the underlying sections of DNA (Chen et al. 2019).

Agricultural scientists are adopting high-throughput (HTP) phenotyping, precision agriculture, and crop-scouting platforms based on the breakthroughs that are being reported in genomics, sensor technology, remote sensing, robotics, and big data analytics. These technological gains along with the data revolution are ushering in an era of digital agriculture that should greatly enhance the capacity of plant breeders and agronomists (Shakoor et al. 2019) in creating a radical step change toward sustainable agriculture and food systems around the world.

The decreasing cost of generating data signaled the advent of data revolution in plant breeding which resulted in high volumes of data (big data) being generated in a short amount of time. Recent advances in computational power and networks massively increased our ability to access, analyze, and recombine big datasets. With this explosion in data, a new challenge for the management of data to get the right information at the right time arises. In this chapter, the innovations in plant breeding that generate petabytes of data are discussed along with the strategies being employed to manage and use this data.

4.2 Plant Breeding

Plant breeding innovation has an impressive story to tell (Fig. 4.1). Since the first intentional cultivation of plants 12–14,000 years ago, the genetic make-up of plants has been altered for crop improvement and nutritional quality (Eriksson 2019). By domesticating plants for food, humans tried to speed up natural selection but still had to wait for unpredictable natural mutations resulting in beneficial traits and then breed from that plant. This process of artificial selection is categorized as the first phase in the evolution of breeding and named Breeding 1.0 (Fig. 4.1) which began

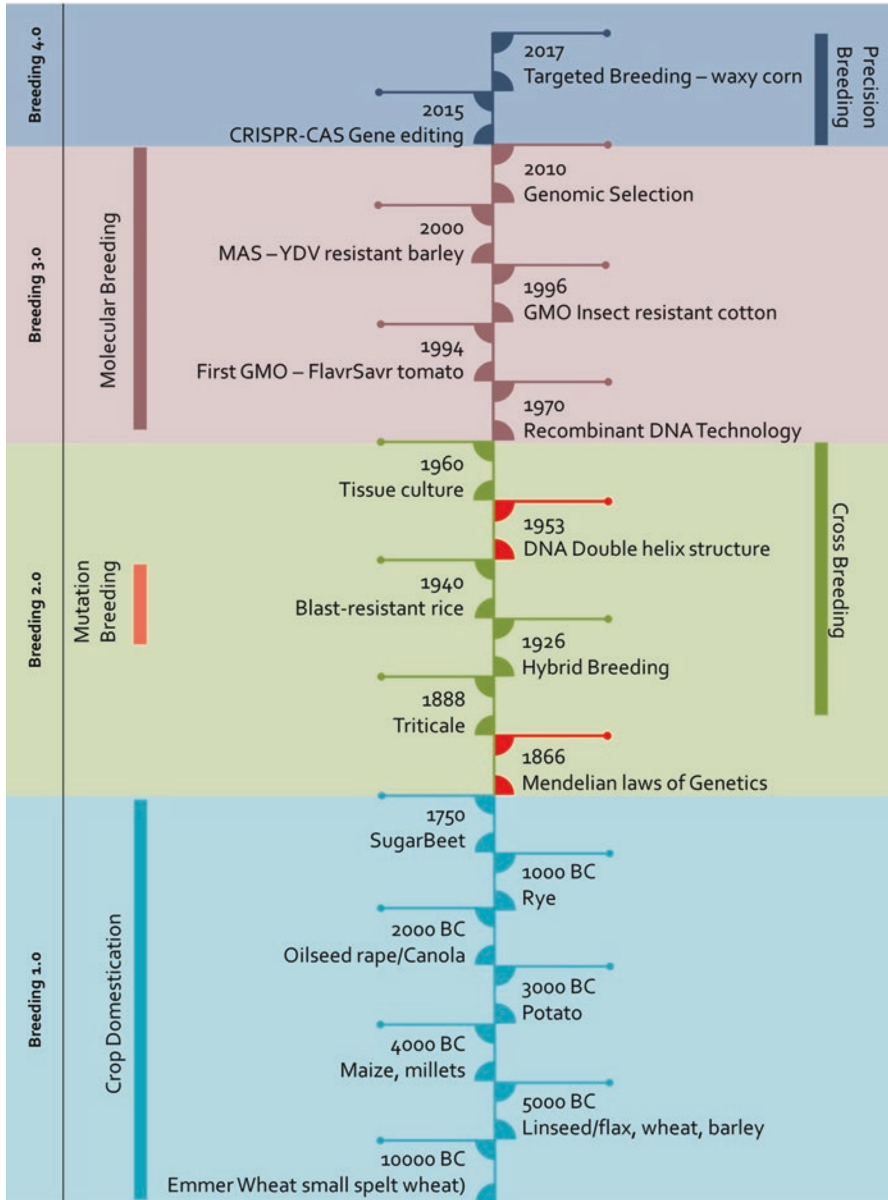

Fig. 4.1 Phases and milestones in plant breeding innovation. The advances in plant breeding are categorized into four phases as described by Wallace et al. (2018) into Breeding 1.0 which refers to the era of artificial selection, Breeding 2.0 which included cross breeding and mutation breeding, Breeding 3.0 consisting of molecular breeding, and the current phase of precision breeding termed as Breeding 4.0

with the cultivation of 7000 species of food plants across the globe (Wallace et al. 2018).

Later, chemicals or radiation were used to accelerate the generation of mutations, but there was still an element of chance because the induction of mutations was random in nature and required screening large number of mutants to be able to identify a few beneficial mutations. This was part of the Breeding 2.0 phase (Fig. 4.1) which began in the late nineteenth century with the rediscovery of Mendelian genetics, understanding of hybrid vigor, and establishment of the quantitative genetics theory (Wallace et al. 2018).

In the 1990s, advances in molecular genomic techniques enabled the use of molecular markers resulting in the era of molecular breeding. With the availability of HTP molecular markers, quantitative trait locus (QTL) mapping became a routine approach for genetic studies of complex traits in plants and is often based on biparental genetic populations (Li et al. 2010). Marker-assisted selection (MAS), which relies on linkage disequilibrium (LD) that exists between the DNA marker and a specific gene, became a popular method of molecular breeding. MAS was highly efficient in gene pyramiding to combine several important genes in one cultivar but was dependent on the availability of large number of DNA markers that cover the whole genome (Ben-Ari and Lavi 2012; Collard and Mackill 2008).

Genome-wide association studies (GWAS) and genomic selection (GS) became promising approaches for genetic improvement of complex traits in crop species due to the decreased cost of genotyping and improved statistical methods. GWAS enabled identification of causal genes for complex traits by using populations of unrelated lines and high-density single nucleotide polymorphism (SNP) markers in different crops. Unlike GWAS, GS used genome-wide markers without identifying a subset of trait-associated markers for marker-based selection by capturing the total genetic variance (Zhang et al. 2016; Wang et al. 2018). This was the phase of Breeding 3.0 (Fig. 4.1) where molecular markers and genomic data began to complement phenotypic data (Wallace et al. 2018).

According to Wallace et al. (2018), we are now on the cusp of Breeding 4.0, a new level of breeding where functional genetic variants can be rationally combined both faster and better than ever before. A directed approach to trait management through targeted genetic alterations is possible due to the advances in recombinant nucleic acid technology. More recently, developments in genome editing provide yet more precision by enabling alterations at exact locations in the genome (Eriksson 2019). Breeding 4.0 is catalyzed by major technological advances like next-generation sequencing and precise modifications to multiple sites in a genome per generation (multiplex gene editing) along with HTP phenotyping which can measure numerous traits with unprecedented spatiotemporal resolution (Wallace et al. 2018).

Technologies in isolation are unlikely to accelerate the rate of genetic gain in crop breeding. However, the successful integration of breeding strategy with the advances in genotyping and phenotyping technologies has the potential to break the stagnant yield barriers in various crops. Integration of “speed breeding” with above approaches could help achieve the 2050 genetic gain targets for the four Fs (food,

feed, fiber, and fuel). Speed breeding refers to shortening of generation time to accelerate breeding and research programs. For example, extended photoperiods and controlled temperature regimes can lead to rapid generation cycling for large-scale application in crop breeding programs (Li et al. 2018).

Efficient use of these technological advances and their integration is dependent on the advances in information systems. The data from these approaches is at a level far beyond what humans can assimilate manually and need systems that can store data in a proper format combined with algorithms that can analyze and interpret results at a massive scale to enable informed decisions. The primary challenge facing plant breeding teams in the 2020s will not be access to modern technology (Cobb et al. 2019), but rather designing systems to handle and analyze the massive amounts of genomic, phenotypic, and environment data that is generated at each stage of the breeding pipeline. The next sections cover the need for data management and state of the art in data storage and analysis with specific reference to plant breeding data.

4.3 Need for Data Management and Integration

Data in science covers what was observed, under which conditions, by whom, when, and how and can be categorized based on these common characteristics. McGilvray (2008), in her book, defined these data categories in a simple manner which we have represented in Fig. 4.2. Irrespective of how it is done, structured or ad hoc, good

Fig. 4.2 Data categories based on type of data and system design. Based on the data type, data can be classified into master, transactional, reference, or metadata. Historical data and temporary data are classes based on the system design. (Adapted from McGilvray 2008)

data management is crucial but not a goal in itself. Rather, data management is the key to knowledge discovery and innovation (Wilkinson et al. 2016). When data is generated, ad hoc management follows, but, with the increase in data volumes and computational power, data management needs a structured approach (Godøy and Saadatnejad 2017). Regardless of the current progress in data management technologies, many challenges remain.

In 2007, Jim Gray, one of the founders of modern database technology and transaction processing, coined the term "data-intensive science" which he described as collaborative, networked, and data-driven. This emerging field of data-intensive science is about exploring and exploiting old data and is described as the fourth paradigm in research after theoretical, empirical, and computational science (Gray and Szalay 2007). The fourth paradigm is integral to the success of modern science and is heavily dependent on a clearly defined data management strategy. In spite of the enormous potential of this approach, data-intensive science has been slow to develop due to the subtleties of databases, schemas, ontologies, and a general lack of understanding of these topics by the scientific community (Bell et al. 2009).

Data management helps manage the information generated by defining strategies, architectures, policies, and procedures to manage data. A strong data management approach includes acquisition, validation, transformation, storage, and protection of data. Data management is an integral part of any project and doubly so for breeding research since a prerequisite for successful plant breeding is the ability to integrate and reuse data for reproducible analytics. This requires standardized data storage ensuring long-term data availability while maintaining intellectual property rights (Köhl and Gremmels 2015). Data management should be incorporated as an essential practice to ensure the success of any breeding program (Fig. 4.3)

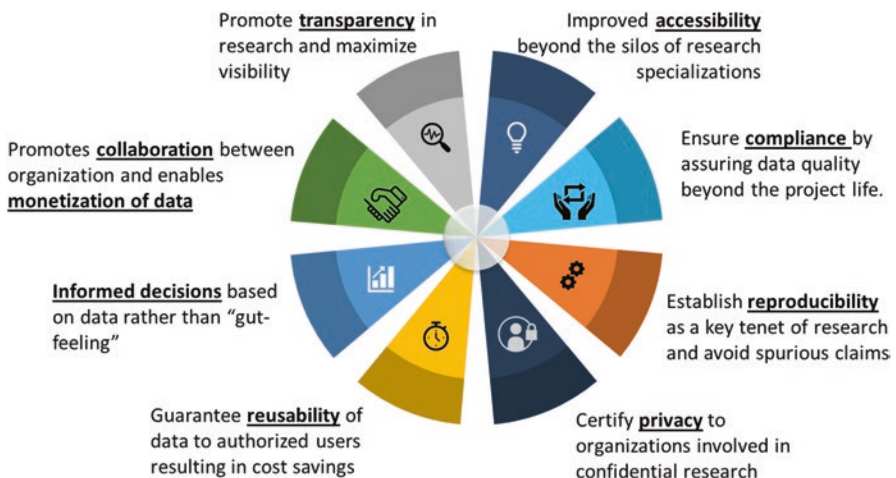

Fig. 4.3 Impact of data management. Having a data management strategy ensures that the data is accessible, reliable, and reusable

since past attempts to modernize breeding without reliable management systems have resulted in failure either due to poor data quality, lack of documentation, or lost institutional memory (Ribaut and Ragot 2019). In the following sections, the key steps in data management with respect to plant breeding are discussed.

4.4 Data Acquisition

The process of collecting data from different sources is called data acquisition. In plant breeding research, the data that is collected is very heterogenous and includes genomic, phenotypic, and environment data. The acquisition and management strategies of these three types of data are discussed in the following sections.

4.4.1 Genotype Data

Genotyping solutions have accelerated the speed and efficiency of plant breeding in fields by enabling selection long before the trait of interest is expressed, thereby saving time, labor, space, and costs in bringing new varieties to the market. These cost savings are significant, especially when plants can be selected at the seedling stage, particularly for traits such as yield and others that are only visible at the time of crop harvesting (Hogers et al. 2018). Variations ranging from a single base pair up to duplications and translocations of entire chromosomal regions can be detected using molecular markers (Patel et al. 2015). With advances in genome sequencing technologies, genetic markers are very popular and used in increasingly diverse and HTP experiments. Next-generation sequencing (NGS) technologies increased the rate of polymorphism discovery, with single nucleotide polymorphisms (SNPs) predominating as the marker of choice, thus enabling the shift to HTP genotyping assays creating massive data outputs (Patel et al. 2015). For instance, the first sequenced maize inbred line, B73, is reported to have a genome size of 2.3 GB (Schnable et al. 2009), and SNPs appear every 44–75 bp on an average (Gore et al. 2009). For precision breeding, the analysis of very large numbers of SNP markers in precisely located single-copy sequences is a prerequisite (Ganal et al. 2011) which results in a high volume of multidimensional data.

A variety of software packages and environments have been developed to allow computation of high-dimensional data. Since each package has its own specialized features, they usually require their own specific input and output formats of data. Table 4.1 summarizes the different types of file formats associated with genotyping data with special reference to SNPs.

As per the global forecast, the global genotyping assay market was valued at approximately USD 11.9 billion in 2018 and is expected to generate around USD 46.9 billion by 2025, at a compound annual growth rate of around 21.8% (Report 2019) suggesting an increasing trend in genotyping data.

Table 4.1 Some commonly used genotype file formats

Format	Comments
PLINK binary format (.bed, .bim, .fam)	The bed file contains the SNP genotypes, coding as AA, AB, BB, or missing, where A is the first allele and B is the second; note that the bed file is stored in binary format; it is not (easily) human readable. The bim file has one row per SNP and six columns, which provide the chromosome, name, genetic, and physical distance of each SNP, as well as the bases corresponding to the 1 and 0 alleles. The fam file has one row per individual and six columns, which provide the individual ID, the family ID, as well as maternal and paternal IDs, sex, and phenotype
GEN (.gen, .gen.gz, sample/.fam, bim)	Contains predictor values, one row per predictor with an optional extra initial column containing chromosomes
BGEN (.bgen)	Binary GEN format
SP (.sp, .bim, .fam)	Sparse partitioning is a raw format where the main data file has one row per predictor and one column per sample. As with PLINK format, the bim and fam files contain details for the predictors and samples, respectively
SPED (.sped, .bim, .fam)	This is the old binary SP format and is same as SP format, except the main data file is in binary format
SPEED (.speed, . bim, .fam)	Binary SP format which is same as old binary SP format, except that the values are stored as truncated float
VCF (.vcf, .vcf.gz)	Variant call format is a compressed text file
SHAPEIT haplotype format	Haplotype pair (=genotype) of each individual forms consecutive columns
IMPUTE allele probabilities format	Is like the SHAPEIT haplotype format but contains a probability for each haplotype
IMPUTE haplotype format	Is similar to SHAPEIT haplotype format
HLAIMP probability format	Splits each HLA locus as a series of biallelic variants
PennCNV/ QuantiSNP format (penncnv)	Uses a single sample per input file
BIMBAM dosage format	Outputs a single column per sample (named by the sample identifier) containing the expected second allele dosage for the sample at each variant

4.4.2 Phenotype Data

Unlike digital genotyping, digital phenotyping is still in its infancy. Limitations on phenotyping efficiency are a key constraint in making genetic advancements in breeding programs (Araus et al. 2018). While biochemical and molecular data play an important role in the evaluation of genotypes for breeding, agronomic data is used as an advancement criterion to bring varieties and hybrids to the market. Nevertheless, the proteome and metabolome of the plant which change in response to genotype-environment interactions (GEI) are a part of phenotype. Central dogma of biology (Crick 1970) states that cells contain hereditary information in the form of DNA (genome), which is transcribed into RNA (transcriptome) and translated into proteins (proteome) that are ultimately involved in the modulation of the metabolome.

4.4.2.1 Proteomic Data

There are two main approaches used in proteomics (study of proteome) based on the objective of the study. For discovery proteomic research requiring a broad range of samples, liquid chromatography-tandem mass spectrometry (LC-MS/MS) in data-dependent acquisition (DDA) mode was routinely used (Hu et al. 2016). In contrast, for targeted proteomics when precise quantification, reproducibility, and validation were the objectives, multiple reaction monitoring (MRM) LC-MS/MS has been the standard. The newer data-independent acquisition (DIA) method approaches the identification breadth of DDA while achieving the reproducible quantification characteristic of MRM but requires complex algorithms to deconvolute the complex spectra (Hu et al. 2016).

4.4.2.2 Metabolomic Data

Metabolomics (study of the metabolome) has made significant progress in the last 2–3 years. Absolute quantitation of metabolites in one-dimensional (1D) 1H nuclear magnetic resonance (NMR) spectra which was done manually in the past can now be automated using new computational approaches (Bingol 2018). These approaches also increase the capability to integrate two-dimensional (2D) NMR metabolomic databases, thereby ensuring consistency in interlaboratory comparisons and accurate identification of metabolites. Hybrid mass spectrometry and NMR approaches facilitate the identification and cataloging of hitherto unknown metabolites (Bingol 2018).

4.4.2.3 Phenomic Data

The slower pace of digitization of phenomic (morphological+ physiological) data defined as the multidimensional phenotypic data at multiple levels of cell, organ, plant, and population (Houle et al. 2010) has created a bottleneck in correlating genotypes with phenotypes. Additionally, the compartmentalization of genomics and phenomics studies has created a gap in the ability to integrate the data across these platforms (Köhl and Gremmels 2015). While large research institutes and corporate research centers have developed HTP phenotyping capabilities, this data cannot be readily generated and used in public research due to the infrastructure cost. Often, phenotyping is followed by conventional data management practices like recording on paper and transferring to MS Excel like storage systems. This is an inefficient and error-prone process. A prerequisite to create data with high statistical power is the integration of datasets from different sources. This integration could be hampered by manual phenotyping since manual efforts are often subjective. Further, quality phenotyping is also required to evaluate the results of mutagenesis, genetically modified organisms, or even CRISPR/Cas9 systems (Araus et al. 2018).

Crop phenomics facilitates the exploration of a gamut of phenotypic information of crop growth in a complex environment. It is an integration of agronomy, life sciences, information science, mathematics, engineering sciences, high-performance computing, and artificial intelligence technology (Zhao et al. 2019). Based on the spatial and temporal resolution at which phenotyping is done, it can be divided into the following:

1. Micro-phenotyping—higher spatial and temporal resolutions of tissue and cells. It is done using high-resolution images combined with automated image analysis which are still complex techniques and not easily available (Araus et al. 2018; Zhao et al. 2019).
2. Organ-level phenotyping—includes the frequently used phenotyping index, such as leaf length, leaf area, and fruit volume. Low-cost 2D cameras provided an effective solution to track growth of plants, but one dimension was lost, and some morphological traits needed further calibration. A cost-effective alternative was the multi-view stereo (MVS) approach where multi-view images and motion techniques were used to extract organ-specific information (Araus et al. 2018; Zhao et al. 2019).
3. Automated phenotyping in controlled environment—phenotyping under controlled environment conditions such as in greenhouses and poly-houses using robotics, sensors, and imaging techniques enabled HTP plant phenotyping but was not cost-effective. Moreover, the controlled nature may not fully replicate the environmental variables influencing complex traits, and this method does not adequately represent GEI at the scale of large landscapes experiencing climate variability with large numbers of progenies being tested by breeding programs (Araus et al. 2018; Zhao et al. 2019).
4. HTP phenotyping in field—in recent years, field phenotyping was done using ground-wheeled, rigid motorized gantry or aerial vehicles (both manned and unmanned remote sensing platforms), combined with a wide range of cameras, sensors, and high-performance computing, to capture phenotyping data with spatial, spectral, and temporal resolutions (Zhao et al. 2019). Integration of ground-based platforms with aerial solutions offers a higher flexibility for phenotyping. For example, sorghum complex trait assessment by Potgieter et al. and Furbank et al. used this approach (Potgieter et al. 2018; Furbank et al. 2019).

Collection of phenotypic data is only the first step in a complex multistage process. Since phenomic experiments are not directly reproducible due to multi-model sensors, differing scales, and environmental variations, the collected data must be stored and translated with high efficiency and accuracy to be used for meta-analyses (Zhao et al. 2019). The major challenge in the use of these HTP phenotyping platforms is data management including metadata collection and data annotation. Additionally, data management gains even more importance in the context of community integration aimed at sharing information and collaboration across organizations. Prominent among the international initiatives that foster cooperation is the EMPHASIS project (the European Infrastructure for Multi-scale Plant Phenomics

and Simulation: https://emphasis.plant-phenotyping.eu/). With the intent of enhancing the ability of machines to automatically find and use the data, in addition to supporting its reuse by humans, Wilkinson et al. (2016) proposed the FAIR principles of data management and stewardship. FAIR stands for the four foundational principles of findability, accessibility, interoperability, and reusability. While accessibility is defined by the organization conducting research, the recommendations of Krajewski et al. (2015) can be used to address the challenges of findability, reusability, and interoperability specific to phenotypic data management:

1. Minimum information (MI): a checklist of attributes that is necessary to describe any experiment to ensure findability. Numerous MI initiatives are registered at the FAIRsharing web portal (https://www.FAIRsharing.org) (Sansone et al. 2019).
2. Annotation with common public ontologies to ensure reusability.
3. Ensure proper use and interoperability of datasets by choosing the proper format (CSV, XML, RDF, MAGE-TAB, etc.).

To use phenomic data effectively, it is important to distinguish the causal intermediates from correlative associations between environment and genotype. Most yield traits are quantitative and phenotypically plastic, and different parts of the genome can be involved in shaping the trait under different environmental conditions. Knowledge of the environment where the crop is grown and GEI helps in selecting genotypes suited for local environments (Mir et al. 2012). The next section provides the details of acquisition of environment data.

4.4.3 Environment Data

Changes in the relative performance of genotypes across different environments are referred to as genotype-environment interactions (GEI) (Baye et al. 2011). A change in the phenotype expressed by a single genotype in different environments is termed as phenotype plasticity (Gratani 2014) and is the result of complex GEI. This plasticity of the phenotype, which enables adaptation to a given environment, can make these plants more resilient against the next limitation at a later stage of its life. For instance, root system architecture modification in response to drought during seedling stage can help the plant during drought in later stages of its life (Pieruschka and Schurr 2019). Environmental effects can also result in heritable variations in genotypes by triggering epigenetic variations like DNA methylation and histone modifications (Duncan et al. 2014). An example is change in seed composition or quality in response to environmental cues that affected mother plants during seed filling (Pieruschka and Schurr 2019). The probability of impact of epigenetic variations on phenotypic plasticity and acquired traits is dependent on transgenerational inheritance of these epigenetic marks (Duncan et al. 2014).

Based on the extent, location, and constitution, environmental factors can be:

1. Micro (unique to a single plant or to a small group of plants, e.g., plant spacing) or macro (are common to a given location at a given time, e.g., average rainfall)
2. Internal (mainly the vacuole content) or external (classified as climate, soil factors, biotic factors, and crop management or cropping system)

Where a plant can grow is determined by climate factors, while other environmental factors determine how a plant grows (Xu 2016; Nicotra et al. 2010). Biotic factors include companion organisms that are beneficial, pests, and pathogens. Crop management, as a unique environment component, involves intercropping, crop rotation, and agronomic practices. Environmental factors that affect plant growth and yield can be modified or dramatically changed by human activities. Xu (2016) proposed the concept of "envirotyping" (complementary to genotyping and phenotyping) to decipher the impact of environment on crop plants. Envirotyping contributes to crop modelling and phenotype prediction while accounting for crop management as well.

For the interpretation of GEI, assessment of detailed environmental conditions is a key element in multidimensional phenotyping. Environmental conditions are generally under tight control in growth rooms and, to a lesser extent, in glasshouses. Nevertheless, both growth room and glasshouse environments are significantly more stable than the fluctuating environmental conditions that plants experience when subjected to field conditions (Bolger et al. 2019). Consequently, genotypes that perform well in controlled environments do not necessarily perform best in the field. This difference is rather relevant when plants are tested for performance under suboptimal conditions like nutrient deficiency or water shortage. Thus, a comprehensive report of environmental conditions during experiments is of importance under controlled and field conditions (Pieruschka and Schurr 2019). This environment data also enables the comparison of outputs of various experiments and to develop the ideotypes for different environmental scenarios. To make phenomic data FAIR, MIAPPE recommendations (i.e., required Minimal Information About Plant Phenotyping Experiments) necessitate a proper description of all necessary metadata, including the environment (Krajewski et al. 2015).

Since environment factors are captured in concert with phenotype data, technological advancements in this field have lagged considerably. Additional factors affecting progress in measurement of environmental factors are that (1) environmental factors have been largely considered as a whole, (2) only major environment factors are measured for the whole experiment/trial, and (3) most environmental factors are dynamic and constantly changing throughout the plant growing period (Xu 2016). To achieve significant progress in using environment data, complex environments need to be divided into individual factors and measured for each plant individually, at every development stage, like the dissection of quantitative traits into individual Mendelian factors.

4.5 Data Integration

Technological advances in molecular and genomic approaches have enabled determination of genic factors and their response to different environments making crop breeding, data intensive. During phenotyping, capturing environment factors provides a context to the phenotype. HTP genotyping generates petabytes of data, and this volume is threefold if not more when phenotype and environment are included. Once data acquisition is complete, the next step is to integrate the data, analyze it, and interpret the results.

To effectively model crop performance under different environments, genomic data has to be integrated with HTP precision phenotype data and corresponding environment variables. Phenotypic and environment information collected for the same set of genotypes will greatly contribute to crop modelling and phenotype prediction by complementary and comparison analyses. While integration of different datasets provides numerous advantages, the challenges lie in the cost of these phenotyping and envirotyping HTP technologies, the scale of genotypes that need to be tested under different environments, and the volume of data that has to be captured across nearly all of the plant developmental stages (Xu 2016).

The role of controlled vocabularies and ontologies in facilitating the integration of different layers of plant breeding data cannot be emphasized enough. Breeding 3.0 and Breeding 4.0 are based on the integration of different layers of genomic and phenotypic data. Traits lie at the intersection of these layers (Shrestha et al. 2012). Capturing phenotypic data and annotating traits manually present an obstacle to data integration and sharing due to the nonstandard subjective nature of manual efforts. Controlled vocabularies or ontologies help overcome this obstacle by ensuring harmonization of vocabulary across experiments (Arnaud et al. 2012). Crop ontology (CO) provides validated trait names for harmonizing the annotation of phenotypic and genotypic data, thereby supporting data accessibility and discovery (Shrestha et al. 2012). Similarly, using standardized ontologies for environment variables, sequence annotations, and gene functions (Table 4.2) can have a huge impact in data integration and discovery through sharing.

Data management becomes a prerequisite to manage huge datasets and use them appropriately in decision-making. Data Management Analysis and Decision Support Tools (DMASTs) are required for a range of operations including planning breeding experiments; maintaining pedigrees; storing and retrieving genotype, phenotype,

Table 4.2 Ontologies of importance in integration of plant breeding data

Ontology name	Link
Environment ontology	https://www.ebi.ac.uk/ols/ontologies/envo
Sequence ontology	http://www.sequenceontology.org/
Crop ontology	https://www.cropontology.org/about
Plant trait ontology	https://www.ebi.ac.uk/ols/ontologies/to
Gene ontology terms	http://www.geneontology.org

and associated environment; and analyzing the integrated data. Integrated pipelines that combine various specialized software play an important role in breeding programs. These analytical pipelines are not only capable of combining genotype, phenotype, and environment data but also help with analysis. There are plenty of free and open-source tools that are available for performing these data analyses. R (https://www.r-project.org/) and Python (https://www.python.org/) are two commonly used open-source, versatile environments for statistical computing and visualization (Rathore et al. 2018). These are comparable to commercially available environments in performance and are modular which enables sharing of packages and "plug and play" applications like PBTools (http://bbi.irri.org/products). To help future plant breeding efforts, a list of tools (both open-source and commercial) based on the works of Rathore et al. (2018) and Varshney et al. (2016) is compiled in Tables 4.3, 4.4, and 4.5.

The need for integration of different types of data for successful breeding experiments has been emphasized by many authors as described above. Another equally important though often undervalued aspect of data management is its impact on community integration. Community integration in this context refers to pooling breeding data sources across the globe. Yu et al. (2016) demonstrated the power of community integration in their study where they used data from gene banks to build a model that predicts the traits associated with sorghum accessions.

4.6 Data Analysis

Recently, data analytics was classified into five different types based on the objective of the analysis (Brodsky et al. 2017). Though this classification was largely in the context of business intelligence, we believe that it can be correlated to the phases of breeding pipeline as shown in Fig. 4.4. In addition to the technological advances in breeding approaches itself, growth of computational power is a huge factor in the success of the current phase of breeding.

The incredible progress in data analytics and artificial intelligence technologies in the last decade has had a profound impact on breeding operations. With cloud computing becoming accessible and more importantly affordable, staggering amounts of data can be stored, integrated, and analyzed with relative ease. Combined with the increased understanding and popularity of artificial intelligence, it is now possible to make higher accuracy predictions in breeding programs as demonstrated by Yu et al. (2016). The scale of current breeding and field research efforts makes it virtually impossible for a human being to manually sift through the data to make informed decisions. To set better context to the discussion in the following sections, Table 4.6 gives a glossary of some data analytic terminology commonly used in breeding analytics, and the following sections detail some examples of analytics in plant breeding.

Table 4.3 Data analysis and visualization tools for genomic data

Purpose	Tool	Link
Genetic maps	MSTmap	http://mstmap.org/
	MadMapper	cgpdb.ucdavis.edu/XLinkage/MadMapper/
	CMap	https://www.broadinstitute.org/connectivity-map-cmap
	MapChart	https://www.wur.nl/en/show/Mapchart.htm
QTL analysis	MAPMAKER	https://omictools.com/mapmaker-tool
	QTL Cartographer	statgen.ncsu.edu/qtlcart/manual
	QGene	www.qgene.org
	QTLNetwork	https://omictools.com/qtlnetwork-tool
	IciMapping	https://www.integratedbreeding.net/386/breeding-services/more...tools/icimapping
Germplasm analysis	STRUCTURE	http://pritchardlab.stanford.edu/structure.html
	EIGENSOFT	https://reich.hms.harvard.edu/software
	Bayesian Analysis of Population Structure (BAPS)	www.helsinki.fi/bsg/software/BAPS/
	Trait Analysis by aSSociation, Evolution, and Linkage (TASSEL)	https://groups.google.com/d/forum/tassel
	PLINK	zzz.bwh.harvard.edu/plink/
Diversity/phylogenetic analysis	NTSYS	www.exetersoftware.com/downloads/ntsysguide.pdf
	MEGA7 (Molecular Evolutionary Genetics Analysis)	https://www.megasoftware.net/
	DARwin	http://darwin.cirad.fr/
	DAMBE (data analysis in molecular biology and evolution)	dambe.bio.uottawa.ca/DAMBE/dambe.aspx
	PAUP (phylogenetic analysis using parsimony)	http://paup.csit.fsu.edu/
Population genetics	GENEPOP	https://omictools.com/genepop-tool
	Arlequin	cmpg.unibe.ch/software/arlequin35/
	PowerMarker	https://omictools.com/powermarker-tool
	DnaSP v5 (DNA Sequence Polymorphism)	www.ub.edu/dnasp/
	SMOGD (Software for the Measurement of Genetic Diversity)	https://scinapse.io/papers/1990337934
	GenAlEx	biology-assets.anu.edu.au/GenAlEx/

(continued)

Table 4.3 (continued)

Purpose	Tool	Link
Molecular breeding	GGT (graphical genotype)	https://articles.extension.org/pages/32462/graphical-genotyping
	Flapjack	https://ics.hutton.ac.uk/wibblewobble/
	iMAS	http://www.icrisat.org/bt-biomatrics-imas.htm
	Integrated SNP Mining and Utilization (ISMU)	https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0101754
	Marker-assisted Back-crossing Tool (MABT)	https://www.integratedbreeding.net/ib-tools/breeding-decision/marker-assisted-back-crossing-tool
	OptiMAS	https://www.integratedbreeding.net/645/breeding-services/bms-plugins/optimas
	solGS	http://cassavabase.org/solgs

4.6.1 Genotypic Analysis

Better statistical models for ordinal phenotypes to improve the accuracy of selection of candidate genotypes are required to overcome the challenge of stagnant genetic gain (Montesinos-Lopez et al. 2019). Breeding 4.0 uses machine learning methods, making efficient use of sequence and image data, in particular neural networks such as convolutional neural networks (CNN) or recurrent neural networks (RNN), which are appropriate for estimating the effects of polymorphisms on endophenotypes (from DNA sequences) or analyzing HTP phenotyping data for predicting component traits (Ramstein et al. 2019).

A benchmarking of two popular machine learning (ML) methods—multilayer perceptron (MLP) and support vector machine (SVM) vs. the Bayesian threshold genomic best linear unbiased prediction (TGBLUP) model for genomic-based prediction performance—revealed that TGBLUP was better. However, as far as computation time is concerned, SVM was the efficient algorithm (Montesinos-Lopez et al. 2019). There is a huge potential for ML approaches in unbiased trait selection and cultivar classification based on small, complex phenotypic datasets derived from pot experiments (Zhao et al. 2016).

Deep learning has been applied in genomic selection (GS) to accurately select individuals with desired phenotypic attributes based on genotypic data. DeepGS predicts phenotypes from genotypes using a deep CNN by learning complex relationships between genotypes and phenotypes from the training dataset (Ma et al. 2018). DeepSort, another deep CNN, can discriminate the embryo regions traditionally used to (manually) discriminate haploids from diploids (Veeramani et al. 2018). These prediction models can help scientists in different disciplines to develop drought and heat-tolerant plants by exploiting desirable GEI (Crossa et al. 2017).

A ML classifier, based on a dataset of deleterious and neutral mutations in *Arabidopsis thaliana*, has been used to predict deleterious mutations in other crops like rice (*Oryza sativa*) and pea (*Pisum sativum*). This approach used different ML

Table 4.4 Data analysis and visualization tools for proteomic and metabolomic data

Purpose	Tool	Link
Metabolomic analysis	BioCyc	http://biocyc.org
	iPath	http://pathways.embl.de
	KaPPA-View	http://kpv.kazusa.or.jp/en/
	KEGG	http://www.genome.jp/kegg/pathway.html
	MapMan	http://mapman.gabipd.org/web/guest/mapman
	MetabolomeExpress	https://www.metabolome-express.org/
	MetaboAnalyst	http://www.metaboanalyst.ca/faces/home.xhtml
	Metscape	http://metscape.ncibi.org
	MGV	http://www.microarray-analysis.org/mayday
	Paintomics	http://www.paintomics.org
	Pathos	http://motif.gla.ac.uk/Pathos/
	PathVisio	http://www.pathvisio.org/
	PRIMe	http://prime.psc.riken.jp/
	ProMeTra	http://www.cebitec.uni-bielefeld.de/groups/brf/software/prometra_info/
	Reactome	http://www.reactome.org
	VANTED	http://vanted.ipk-gatersleben.de
	MetPA	http://metpa.metabolomics.ca
Proteomic analysis	PRoteomics IDEntifications database (PRIDE)	http://www.ebi.ac.uk/pride
	MSDA	https://msda.unistra.fr/
	COMPASS	https://github.com/dbaileychess/Compass
	PICR	http://www.ebi.ac.uk/Tools/picr/
	CRONOS [33]	
	Mascot	http://www.matrixscience.com
	SEQUEST	https://omictools.com/sequest-tool
	UniProt knowledgebase	www.uniprot.org/help/uniprotkb
	Ensembl	www.ensembl.org/
	KEGG	www.genome.jp/kegg/pathway.html
	Reactome	http://www.reactome.org
	Ingenuity Pathway Knowledge Base	https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/
	PANTHER	http://pantherdb.org/about.jsp
	BioGRID	https://thebiogrid.org/
	IntAct	http://www.ebi.ac.uk/intact.
	STRING	https://string-db.org/
	Cytoscape	www.cytoscape.org/
	NCI pathways	http://www.wikipathways.org/index.php/WikiPathways
	EnrichNet	www.enrichnet.org/

(continued)

Table 4.4 (continued)

Purpose	Tool	Link
Annotation tools	Pfam	http://pfam.xfam.org/
	InterPro	https://www.ebi.ac.uk/interpro/
	SMART	http://smart.embl-heidelberg.de/
	DAVID	https://david.ncifcrf.gov/

Table 4.5 Data integration, storage, and analysis tools

Purpose	Tool	Link
Data analysis	ASReml	https://www.vsni.co.uk
	Genstat	https://www.vsni.co.uk/software/genstat/
	MINITAB, Statistical Package for Social Sciences	http://www.spss.co.in/
	Statistical Analysis System	https://www.sas.com
	Statistica	https://software.dell.com/products/statistica/
	STATA	www.stata.com/
Integrated pipeline	International Crop Information System (ICIS)	www.icis.cgiar.org
	Integrated Breeding Platform (IBP)	http://www.integratedbreeding.net
	Breeding4Rice (B4R)	https://breeding4rice.irri.org/
	CassavaBase	https://www.cassavabase.org/
	Katmandoo	http://www.katmandoo.org/
	Phenome One platform	http://phenome-networks.com/solutions/for-plant-breeders/
	AGROBASE Generation II	http://www.agronomix.com
	PRISM	http://www.teamcssi.com/index.html
	Progeny	http://www.progeno.net/software
	Progen software	http://www.progeno.net/
	E-Brida	http://www.agripartner.nl/en-us/products/plantbreedingsoftware.aspx
	GeneFlow	http://www.geneflowinc.com
Data storage and sharing platforms	Dataverse	https://dataverse.harvard.edu/
	CKAN	https://ckan.org
	ResearchSpace	https://www.researchspace.com
	e!DAL	https://edal.ipk-gatersleben.de/
	DSpace	http://www.dspace.org
	Fedora	http://fedorarepository.org

models like linear SVM, Gaussian SVM, and random forest classifiers (Kovalev et al. 2018).

A new avenue in intelligent agriculture is determining the important physiological and agronomic traits contributing to yield through ML algorithms (Shekoofa et al. 2014). A preferred ML method is the decision tree model which has the capa-

Descriptive	Diagnostic	Predictive	Prescriptive	Proactive
What is happening?	Why is it happening?	What can happen?	What action should be taken?	What is the best action?
Information		→		Optimization
Hindsight	→	Insight	→	Foresight
Breeding 1.0	Breeding 2.0	Breeding 3.0	Breeding 4.0	Breeding 5.0
Need better food - domesticate	Decipher genetics and GEI	Given an environment, what can be produces?	Precise genome modification for desired effect	De-novo gene and pathway designs

Fig. 4.4 Types of analytics in relation to phases of plant breeding. Breeding 4.0 evolved from predictive analytics to prescriptive in the last decade or so

bility of illustrating different pathways of yield increase in breeding programs (Shekoofa et al. 2014). Artificial neural networks (ANNs) and SVM-based ML models are becoming popular in agriculture production systems. More specifically, ANNs were used in crop, water, and soil management, while SVMs were used for livestock management (Liakos et al. 2018).

4.6.2 Phenotypic Analysis

ML and data-driven approaches play major roles in bridging the gap between genomics and phenomics data (Bolger et al. 2019). Powerful statistical approaches are essential to make use of the increasing amount of phenotyping information and the complex trait sets that describe crop cultivars (Zhao et al. 2016). As discussed earlier, MIAPPE guidelines with a checklist for describing plant phenotyping experiments ensure reusability and integration of phenotypic data with other systems (Bolger et al. 2019). MIAPPE ensures proper description of metadata including the environment and solves the problem of data standardization in experimental factors.

Identification, classification, quantification, and prediction are the four stages of decision-making in plant stress phenotyping and plant breeding activities which utilize ML methods (Singh et al. 2016). ML-based robust extendable frameworks can accurately identify, classify, and quantify abiotic and biotic stresses in HTP manner. A real-time phenotyping framework using ML for plant stress severity rating in soybean investigated ten different classification approaches, with the best classifier being a hierarchical classifier (Naik et al. 2017). Unsupervised identification of visual symptoms including quantitative measure of stress severity in a single ML framework was developed for soybean (Ghosal et al. 2018). Singh et al. (Singh et al. 2016) compiled a comprehensive overview of best-practice guidelines for various stress traits along with a user-friendly taxonomy of ML tools.

With the technological advances in HTP phenotyping relying heavily on images, high-quality, annotated training image datasets are essential for effective analysis. Under challenging conditions such as illumination, complex background, different

Table 4.6 Glossary of data analytic terms used in plant breeding analytics

Term	Definition
API (Application Program Interface)	A set of programming standards and instructions for accessing or building web-based software applications
Application	Software that enables a computer to perform a certain task
Artificial intelligence	Ability of a computing machine to apply insights from previous experience accurately to new situations
Batch processing	Processing high volumes of data where a group of transactions is collected over a period of time. Hadoop is focused on batch data processing
Big data	Is complex data that is huge in size and yet growing exponentially with time
Classifier	An algorithm that classifies observations into different categories
Cloud computing	A distributed computing system hosted and running on remote servers
Cloud	Internet-based application or service that is hosted remotely
Cluster computing	Computing using a "cluster" of pooled resources of multiple servers
Data governance	A set of rules that ensure data integrity
Data integration	The process of combining data from different sources
Data integrity	Refers to the accuracy, completeness, timeliness, and validity of the data
Data lake	A large repository of enterprise-wide data in raw format
Data mining	Finding meaningful patterns and deriving insights in large sets of data
Data modelling	Defines the structure of the data
Dataset	A collection of data, often in tabular form
Data warehouse	A repository for enterprise-wide clean data in a structured format after integration with other sources
Database	A digital collection of data and the structure around which the data is organized
Grid computing	Connecting different computer systems from various locations, often via the cloud, to reach a common goal
Hadoop	An open-source software framework administered by Apache that allows for storage, retrieval, and analysis of very large datasets across clusters of computers
Load balancing	The process of distributing workload across a computer network or computer cluster to optimize performance
Machine learning	A type of artificial intelligence where systems can learn, adjust, and improve based on the data fed to them
Machine-generated data	Data automatically created by machines via sensors or algorithms or any other nonhuman source
Massively parallel processing (MPP)	Using many different processors (or computers) to perform certain computational tasks at the same time
Neural network	Models inspired by the real-life biology of the brain. Deep learning is a similar term and is generally seen as a modern buzzword, rebranding the neural network paradigm for the modern day
Parallel processing	A mode of operation in which a process is split into parts, which are executed simultaneously on different processors attached to the same computer

(continued)

Table 4.6 (continued)

Term	Definition
Predictive modelling	The process of developing a model that will likely predict a trend or outcome
Test dataset	An independent dataset of observations that has the same probability distribution as the training dataset and is used for testing the model
Training dataset	A set of observations used to train a model

resolutions, size, and orientation of real scene images, it becomes difficult to classify image datasets. LeNet architecture as a CNN was effective in classifying such images (Amara et al. 2017). An overview of computer vision-based phenotyping for improvement of plant productivity using ML by Mochida et al. (2019) describes two approaches for taxonomic classification—(1) custom feature based and (2) CNN based. SVM was used in both approaches for taxonomic and physiological state classification.

One critical challenge with all ML algorithms, whether supervised or unsupervised, is that these algorithms require good-quality annotated training data. If the quality of annotation is not good, the model will not be reusable with other data types and scenarios. For example, detection of plant diseases using images requires huge amount of training data of plant images and proper labels for a given set of diseases. Largest collection of annotated image dataset for a single plant disease (maize northern leaf blight) is freely available for building deep learning models at https://osf.io/p67rz/ (Wiesner-Hanks et al. 2018). Another dataset called SPIKE containing hundreds of high-quality images with over 20,000 labeled wheat spikes was used to estimate yield using CNNs, making it a significant advancement in field-based plant phenotyping (Hasan et al. 2018).

The availability of these large-scale plant image datasets combined with effective CNN-based algorithms and the tools available for computer vision-based plant phenotyping have enabled remarkable advancements in plant recognition and taxonomic classification (Mochida et al. 2019). Many ML algorithms including random forest, SVM, vision-based detection, and neural networks have been tried for prediction of plant diseases from a given set of images as listed in Table 4.7. Based on this analysis of literature, deep CNN appears to be the preferred algorithm with AlexNet, GoogLeNet, and CaffeNet being the most popular architectures used for predicting plant diseases. Singh et al. compiled a list of deep learning approaches in plant stress image-based phenotyping while comparing deep learning tools against other techniques to assess decision accuracy, data size requirement, and applicability (Singh et al. 2018).

Models using CNN and semisupervised methods were trained to classify 57 different combinations of crop species and diseases (Cortes 2017). In a survey of 40 research papers, it was demonstrated that deep learning algorithms such as CNN provide high accuracy, outperforming existing commonly used image processing techniques with respect to differences in classification or regression performance (Kamilaris and Prenafeta-Boldú 2018). To identify the type of disease in cotton

Table 4.7 Compilation of published ML packages/tools used for disease prediction in plants

Crop	Disease	Method	Package/tool	References
Grapefruit peel and sugar beet leaves	Bacteria, fungi, and viruses	Vision-based detection algorithm		Dhaygude and Kumbhar (2013)
Plant village dataset, representing every crop-disease pair	Neural network learns the "notion" of plant diseases or learning the inherent biases in the dataset	Deep CNN	AlexNet and GoogLeNet architecture	Mohanty et al. (2016)
Random selection	Detects leaf presence and distinguishes between healthy leaves and 13 different diseases	Deep CNN	CaffeNet architecture	Sladojevic et al. (2016)
Random selection	Many diseases	NN	Genetic algorithm, SVM, PCA, KNN classifier	Dhygude and Kumbhar (2013)
Banana, beans, lemon, rose	Bacterial disease in rose and beans leaf, sun burn disease in lemon leaf, early scorch disease in banana leaf, late scorch disease in beans leaf, fungal disease in bean leaf	Vision-based detection algorithm		Arivazhagan et al. (2013)
Cassava	Cassava brown streak disease, cassava mosaic disease, brown leaf spot, red mite damage, and green mite damage	Deep CNN		Ramcharan et al. (2017)
Plant village dataset	Plant disease severity	Deep CNN	VGG16, VGG19, Inception-v3, and resNet50	Wang et al. (2017)
Tomato	Canker, gray mold, leaf mold, low temperature, miner, nutritional excess, plague, powdery mildew, whitefly	CNN	R-CNN, R-FCN, VGG-16	Fuentes et al. (2017)
Cotton leaf	Bacterial blight, Fusarium wilt, leaf blight, root rot, micronutrient, verticillium wilt	Neural network (NN)	Cross Information Gain Deep forward NN	Revathi and Hemalatha (2014)

(continued)

Table 4.7 (continued)

Crop	Disease	Method	Package/tool	References
Grape	Black rot, downy mildew, powdery mildew, normal and leaf roll	NN and image processing	Canny edge detection, gray-level co-occurrence matrix (GLCM) algorithm	Kakade and Ahire (2015)
Cotton leaf	Leaf roll, leaf curl, leaf crumple, leaf spot, anthracnose, bacterial blight, crown gall	Image processing	k-means clustering algorithm, gray-level co-occurrence matrix	Pachore et al. (2016)
Tomato and other different plants	Powdery mildew, tomato viruses, fungal pathogen	SVM, Bayesian classifier, and random forest		Yang and Guo (2017)
Pomegranate plant	Foliar disease spots	Image processing	k-means clustering, thresholding-based regionextraction	Naik et al. (2014)
Soybean	Iron deficiency chlorosis (IDC)	SVM, linear discriminant analysis	e1071	Bai et al. (2018)
Maize	Northern leaf blight (NLB)	CNN		Wiesner-Hanks et al. (2018)
Wheat	*Septoria tritici* blotch (STB) disease	Random forest		Odilbekov et al. (2018)
Melon	Diseased leaves	Multiple		Pineda et al. (2018)
Soybean	Soybean charcoal rot disease	3D CNN		Nagasubramanian et al. (2018)

plant, image processing with spatial fuzzy c-means (FCM) and probabilistic neural network (PNN) classifier that effectively segmented the diseased portion of the image was used (Zadokar et al. 2017; Gulve et al. 2015).

A few studies used transfer learning where a model developed for one set of problems was reused as the starting point for another set of problems. For example, CNNs were employed to classify tomato plant leaf images based on the visible effects of diseases. In addition to transfer learning, training a CNN from scratch using the deep residual learning method has also been experimented (Atabay 2017).

4.6.3 Modelling GEI

Modelling of the reaction norms (translation of environmental inputs into phenotypes) for a set of genotypes is a central objective in many breeding and genetic studies. Selection of superior genotypes for a particular environment involves pre-

dicting phenotypic response as a function of genetic and environmental factors (Bustos-Korts et al. 2016).

Nitrogen use efficiency (NUE) is defined as grain yield per unit of nitrogen available in the soil. Selection of maize inbred lines that are superior for NUE is complex, since they are controlled by many genes and are strongly affected by the environment. A novel Bayesian multi-trait multi-environment (MTME) model was used to identify and select for superior inbred lines for nitrogen uptake and utilization efficiency (Torres et al. 2018).

Quantitative relationships of environmental factors and identification of plasticity index over a full range of an environmental factor were done using meta-analysis of specific leaf area (SLA; leaf area/leaf mass ratio) as a phenotypic trait (Poorter et al. 2010). Pattern analysis and quantification of biomass allocation was done using meta-analysis to assess the influence of growth environment, plant size, evolutionary history, and competition in vegetative plants (Poorter et al. 2012). Genetic variability to temperature response which includes tissue expansion, cell division, and plant cycle progression was analyzed in the range of 6–37 °C in different lines of 18 species (17 crop species, different genotypes) via the meta-analysis of literature references (Parent and Tardieu 2012). Meta-analyses often employ genotype imputation which estimates missing genotypes from high probability haplotype or genotype reference panels (Shi et al. 2018).

Multi-trait deep learning (MTDL) models were found to be very competitive for performing predictions in the context of genomic selection, with the important practical advantage that it requires less computational resources than the BMTME model. Bayesian multi-trait and multi-environment (BMTME) model is a multi-trait version of the genomic best linear unbiased prediction (GBLUP) univariate model (Montesinos-López et al. 2018).

Lobell et al. (2011) developed a database of yield response models to evaluate the impact of recent climate trends on major crop yields at country scale for the period 1980–2008. Publicly available datasets on crop production, crop locations, growing seasons, monthly temperature, and precipitation were combined in a panel analysis of four crops (maize, wheat, rice, and soybeans) for all countries in the world. Linking evolutionary game theory (EGT, a mathematical tool to analyze natural selection in situations where organisms interact) with information about physiological regulation of trait expression and ecological interactions in agroecosystems provides a framework to understand plant performance under competition for resources (Anten and Vermeulen 2016).

4.7 Outlook and Future Perspectives

In the era of modern digital agriculture, deep learning and convolutional networks will continue to play an important role in advancing research and product development within the agricultural industry (Veeramani et al. 2018). An optimized precision breeding and crop production system enabling the design of genotypes with an

optimized phenotype for a particular environment can be built up with a four-dimensional (4D) profile, consisting of genotype, phenotype, environment, and developmental stages (Xu 2016). This is the premise behind the next phase of breeding, i.e., Breeding 5.0. A review of basic concepts and procedures of ML applications demonstrated that ML with big data technology would facilitate accelerated research in plant sciences (Ma et al. 2014).

To enter this era of optimized breeding, there is a need to build big data compatible parallel computing and data management infrastructure and at the same time develop new algorithms to get insights from the large amount of data. Additionally, since no one organization can individually generate all the data required for the best decision across a range of environments and crops, global initiatives for community integration gain importance. For the success of such community integration initiatives, it is imperative to adopt proper data management guidelines and strategies that ensure harmonization, integration, and reusability of data.

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Chapter 5
Advanced Quantitative Genetics Technologies for Accelerating Plant Breeding

Dharminder Bhatia

5.1 Introduction: Historical Background

Kempthrome (1957) stated "Plant breeding is an applied quantitative genetics". In fact quantitative genetics is fundamental to plant breeding activities. The branch of quantitative genetics has a long-standing history with its roots tracing back to Darwin's idea of selection as the chief agent of evolutionary change. His ideas provided a framework to visualize the progressions by which complex traits change over time. However at that point how these changes pass from one generation to another was not understood. In 1865, Gregor Mendel established the hereditary principles with his meticulous experiments on pea which went unrecognized up to the 1900s. Between 1865 and 1900, Francis Galton performed experiments to elucidate Mendel's principles of inheritance. The Galton's approach differed from Mendel in the choice of experimental material (particularly quantitative characters), and his observations could not enable him to draw similar conclusions. However, he provided the strong basis to study the quantitative variation. He along with Karl Pearson developed statistical concepts of regression, correlation and multiple regression to study relationship among relatives (Galton 1889; Pearson 1894) and laid the foundation for the branch of quantitative genetics. The results from his experiments were later expanded by Karl Pearson and his associates that fell in controversy with William Bateson and his group who strongly supported Mendel's principles. The interpretations from experiments of Johannsen (1909) and Shull (1908) and experimental evidences in the form of multiple factor hypotheses (Nilsson-Ehle 1909; East 1910) gave proof of involvement of more than one Mendelian factor and environment in the continuous nature of variation for quantitative characters.

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The exemplary paper of Fisher (1918) showed that biometrical results from correlation of relatives can be used to estimate different genetic components of variance and interpret Mendelian inheritance of genes controlling quantitative traits. Using the principle, several mating designs were proposed which were later extensively used in plant breeding programmes (Singh et al. 2004). Of these, biparental populations, polycross, topcross, North Carolina (I, II, III), Diallels (I, II, III, IV) and Line × Tester have been commonly used. The principle behind mating designs was to develop progenies of relatives by making a series of crosses among individuals of a random mating population. The kind of relatives included half-sibs (one parent common), full-sibs (both parent common) and parent-progeny relationships. In addition, mating designs were used for analysis of general and specific combining abilities (GCA and SCA) that helped the breeders to select the parents for hybrid and pure line breeding. Mating designs were an excellent tool to estimate different components of genetic variation of quantitative traits and to generate breeding material in the form of different cross combinations. The information thus generated helped plant breeder to carry forward the breeding material, deploying best possible breeding strategy. However analysis of mating designs is based on several assumptions which practically cannot be always ignored.

Accuracy of selection of quantitative traits is hindered by the effect of environment. Originally defined by Lush (1935), breeder's equation helped the plant breeders to know the effectiveness of selection of quantitative traits. A simple, but effective equation "$R = h^2S$" determines the response to selection (R) which is a property of heritability (h^2) and selection differential (S). Breeder's equation was further extended to get $R = \Delta G = ir_A\sigma_A/Y$ (Eberhart 1970), where ΔG is genetic gains over time, "I" is selection intensity, r_A is the accuracy of selection, σ_A is the additive genetic variation and "Y" is the number of years per cycle. It is obvious from the equation that efficiency of response to selection or genetic gains for quantitative traits is a product of selection intensity, selection accuracy and additive genetic variance. Selection intensity depends upon the population size, and breeder has limited opportunity to increase selection intensity. However the accuracy of selection requires more precise phenotyping in multiple environments.

The statistical tools allowed plant breeder to estimate genetic components of variation, deciding breeding strategies and predicting gains from selections. However, these tools were inefficient in estimating the precise genetic control of quantitative traits especially in terms of number and location of genes controlling quantitative traits and their mechanisms thereof. Additionally, the breeding for quantitative traits tends to be less efficient and time-consuming possibly due to complexity of these traits and nonavailability of tools for their directed manipulations.

5.2 Molecular Markers: Resurgence of Quantitative Genetics

The recognition of chromosomal theory of inheritance and phenomenon of linkage and crossing over in 1920 made possible to determine the location of genes controlling quantitative traits on the chromosomes, estimating their effect and tracing inheritance in the progenies. Using the phenomenon, the first report of mapping a quantitative trait (seed weight) in common bean was given by Sax (1923) using morphological marker (seed colour). The study encouraged quantitative geneticists to use such approach to study quantitative traits (Thoday 1961). However, fewer number of morphological markers was a major limitation. Besides these markers could not precisely estimate the effect of linked polygenes due to its own major effect (Tanksley 1993). The advent of DNA-based molecular markers opened up new possibilities to precisely locate these genes on the chromosomes. The DNA segments that govern a quantitative trait are called quantitative trait loci (QTL) by Geldermann (1975). Being phenotypically neutral, molecular markers are efficient in estimating the effect of QTL (Tanksley 1993).

The development of hybridization-based RFLP (restriction fragment length polymorphism) as first molecular markers (Botstein et al. 1980) sufficed the limitation of fewer number of markers. The use of RFLP and QTL detection using interval mapping approach (Lander and Botstein 1989) built a framework for understanding genetic control of quantitative traits. Along with RFLP, innovation of DNA sequencing technologies (Sanger and Coulson 1975) and PCR (polymerase chain reaction)-based molecular markers such as SSR (simple sequence repeats) (Litt and Luty 1989) helped the quantitative geneticists to scan of the whole genome allowing fine resolution mapping and cloning of QTL. The cloning of first gene underlying QTL governing fruit size in tomato (Frary et al. 2000) commanded the researchers for QTL mapping studies in plants and animals for a wide range of phenotypic traits. The development of various statistical packages such as MapMaker/QTL (Lander et al. 1987), QGene (Nelson 1997), PLABQTL (Utz and Melchinger 1996), QTL Cartographer (Wang et al. 2012), QTLNetwork (Yang et al. 2008), *R*/QTL (Broman et al. 2003), etc. provided sophisticated platform for identification of QTL and their contribution to the total phenotypic variation.

In general, the QTL mapping studies used classical linkage mapping and statistical analysis in segregating populations to dissect the genetic architecture of quantitative traits. The basic principle of QTL mapping is to classify individuals in mapping populations into marker genotypic classes based on presence and absence of particular marker locus and to determine whether significant differences exist between classes with respect to the quantitative trait under investigation (Collard et al. 2005). It involved (1) development of biparental populations such as F_2/F_3, RILs (recombinant inbred lines), doubled haploid (DH) and backcross populations (BC); (2) genotyping the population with genome-wide distributed molecular markers and development of dense molecular maps; (3) phenotyping the population in multi-environments depending upon complexity of trait; and (4) application of statistical platforms to identify QTL. Identification of QTL follows fine mapping of

individual QTL with the development of QTL-NILs (near isogenic lines). The process is referred as Mendelizing QTL (Salvi and Tuberosa 2007). For development of QTL-NILs, plant possessing target phenotype with minimum size of donor segment at QTL region and minimum number of residual donor segments throughout the genome is selected and backcrossed three to four times with recurrent parent followed by continuous selfing.

QTL mapping studies flourished in most of the important crops with the development of biparental mapping population such as RILs, crop-specific molecular markers such as SSR and several breeder-friendly statistical packages. These developments resulted in identification of thousands of QTL for several important traits. However, the pace of QTL detection for most of the important traits was still going at sluggish rate mainly due to time involved in generating genotyping data using PCR-based markers and developing suitable mapping populations. In addition, utilization of these QTL in mainstream crop improvement programme was almost negligible probably due to non-validation of most of the QTL in QTL mapping experiments. In addition, insufficient genome coverage with SSR markers, lesser precision in generating phenotypes, inconsistency in QTL detection across populations and genetic architecture of quantitative traits probably resulted in detection of several false-positive QTL (Holland 2007). The identification of candidate genes and cloning of gene underlying QTL were a long-lasting procedure and a bottleneck in understanding the genetic architecture of quantitative traits.

5.3 Advances in Quantitative Genetics

Past one and half decade has seen several advancements including high-throughput genotyping and phenotyping procedures, finished whole genome sequence of important crops, advanced mapping populations with high recombination rates and strategies for rapid generation advancements that accelerated understanding of several quantitative traits and their precise utilization in crop improvement programmes.

5.3.1 High-Throughput Genotyping Procedures

The advent of sequencing technologies led to identification of variation at the single base pair level referred as single nucleotide polymorphism (SNP). SNPs are the most abundant DNA markers that are evenly distributed and can tag almost any gene or locus on a genome (Brookes 1999). The introduction of sequencing platforms based on Sanger di-deoxy sequencing method and start of genome and expressed sequence tags (EST) sequencing projects accelerated identification of SNP markers by comparison of orthologous sequences (Wang et al. 1998). These

developments gave rise to a new era of genomics that contributed the finished whole genome of human, *Arabidopsis*, rice and several microorganisms. However, high cost and low throughput of generated sequence were the major limitations of this method. In 2005, Roche released the first next-generation sequencing (NGS) platform "454 GS20" that significantly reduced the cost and increased throughput. Thereafter emergence of several NGS platforms including Illumina HiSeq 2500, ABI SOLiD 5500xl, Ion Torrent, PacBio RS and Oxford Nanopore and development of several bioinformatic tools simplified the process of discovery of SNPs and INDELs (insertions/deletions) in a more efficient, cost- and time-saving manner (Chen et al. 2013).

The NGS-based platforms gave birth to reduced representation sequencing and whole genome resequencing-based high-throughput genotyping approaches, where the entire genome of a number of individuals in a population are sequenced to capture genome-wide SNP markers. Genotyping by sequencing (GBS) is currently a powerful NGS-based reduced representation sequencing technique for genotyping large populations (Bhatia et al. 2013). Genotyping with SSR markers takes weeks and months to complete depending upon size of population and number of markers, whereas GBS can complete a whole process of genotyping in 2–3 weeks irrespective of population size besides generating thousands of markers (Spindel et al. 2013; Bhatia et al. 2018). In general, GBS is sequencing of fragments generated through digestion of genome with restriction enzymes on NGS platform. The choice of restriction enzymes depends upon complexity and size of genome.

GBS has evolved into different versions such as RAD-seq (restriction site associated DNA sequencing), ddRAD-seq (double-digest restriction site associated DNA sequencing), Rest-seq (restriction fragment sequencing), SLAF-seq (specific-locus amplified fragment sequencing) and Skim-GBS (skim-based genotyping by sequencing) (Bhatia et al. 2013; Sun et al. 2013; Golicz et al. 2015). These versions differ based on use of size selection, extent of complexity reduction and genome coverage. Since GBS is a population-based genotyping procedure, low-depth sequencing is followed to make it cost-effective which is responsible for high rate of missing data. The low-depth sequencing makes it an ineffective genotyping approach in heterozygous populations. Due to reduced representation sequencing, GBS has low genome coverage.

Whole genome resequencing (WGR) overcomes the limitation of missing data point and heterozygous calls due to high coverage and depth. In general, WGR is sequencing of enough DNA fragments (>20X) to cover the whole genome of an organism. GBS and WGR has been used in several studies for genotyping mapping populations for construction of high-density linkage maps and mapping QTL, linkage and genome-wide association studies (GWAS), improvement of reference genomes and genomic selection (Poland and Rife 2012; Bhatia et al. 2013; Chung et al. 2017; Nguyen et al. 2018).

5.3.2 High-Throughput Phenotyping Procedures

Accuracy to study quantitative traits depends entirely on robust and accurate phenotyping procedures. Traditional phenotyping procedures are labour intensive, time-consuming and invasive. These are less efficient and erroneous particularly for traits affected by the environment. Limited efficiency of these procedures is increasingly being perceived as a bottleneck in accelerating breeding programmes particularly with advances in high-throughput genotyping (Araus et al. 2018). Realizing the need for precise and rapid phenotyping of traits, several high-throughput phenotyping platforms have been developed in the past (Zhao et al. 2019). These platforms are based on noninvasive image analysis which uses digital (red-green-blue) cameras to capture high-resolution images both in controlled and natural environmental conditions facilitating faster, dynamic and precise data points. The images are analysed by computer and machine learning methods to extract trait information. Different remote sensing technologies such as visible imaging, multispectral and hyperspectral fluorescence, thermal infrared imaging, 3D imaging and tomographic imaging are used to collect data of complex quantitative traits related to the plant growth, yield and adaptation to biotic or abiotic stress (Li et al. 2014). The remote sensing tools are fixed at a particular position or carried to different places in the field with the help of mobile phenotyping platforms such as drones, carts, tractor-based systems, etc.

Since phenotyping has a wider scale than genotyping, an ideal high-throughput phenotyping platform should be flexible and cost-effective and should have user-friendly applications for data management and analysis. In addition, results from controlled environment platforms should replicate in target field conditions. Due to high initial investments, establishing these facilities at particular locations has been given emphasis in the past. As an example, a plant-to-sensor platform (LemnaTec Greenhouse Scanalyzer) has been established at the Indian Agricultural Research Institute, New Delhi. The largest robotic field phenotyping platform has been established at Maricopa, USA.

High-throughput crop genotyping and phenotyping procedures provide means to accelerate trait dissection and gene discovery and improve selection accuracy and selection intensity. Another important invention in the recent years is “speed breeding” that refers to shortening the life cycle of crop plants by using extended photoperiod and controlled temperature (Watson et al. 2018). In a recent review, Hickey et al. (2019) have discussed the potential of all these developments in manipulating quantitative traits and developing better crop plants to feed the ever-growing population.

5.3.3 Advances in QTL Mapping

Conventional QTL mappingprocedures rely on development of homozygous biparental populations and dense genetic linkage maps which itself are time-consuming and laborious procedures. In addition, the precision of mapping QTL depends upon

accurate and highly saturated genetic maps. Further, genetic maps need to be integrated with physical maps for fine mapping and cloning of QTL. Recent developments in QTL mapping procedures such as GWAS and sequence-based bulked segregant analysis do not require the development of homozygous mapping populations and development of dense genetic maps. Complementing these procedures with high-throughput phenotyping saves time for multi-environment evaluations leading to rapid identification of QTL for important traits.

5.3.3.1 GWAS: Mapping QTL in Natural Populations

QTL detection methods relied heavily on linkage analysis in biparental populations. However, time and cost required to develop biparental populations and limited recombination events in their development are the major limiting factors in high-resolution detection of QTL. In addition, linkage analysis in biparental populations can only capture the allelic variation of two parents. Therefore accuracy of QTL detection depends largely on the selection of parental lines. The recombination events can be increased by several generations of inter-crossing such as generating advanced inter-cross RILs. The allelic diversity in a population can be increased by generating multi-parental populations such as MAGIC (multi-parent advanced generation inter-cross) population (Jacquemin et al. 2013). However developing such kind of population is still labour intensive. Further, biparental populations cannot be developed in the crops which are vegetatively propagated, have crossability barriers and have large generation time. In general, linkage mapping identifies the QTL in a window of 10–20 cM due to limited recombination events. GWAS has emerged as a powerful approach that takes advantage of historical recombination events in natural population resulting in high-resolution mapping of QTL (Zhu et al. 2008). The approach was first used in humans about 10 years ago and found significant success in translating its results to application in therapeutics (Visscher et al. 2017). After its demonstration in crop plants (Thornsberry et al. 2001), GWAS has generated a higher level of interest in the scientific community.

GWAS uses linkage disequilibrium (LD) in a population to find association of phenotype and genotype (Flint-Garcia et al. 2003). In addition, size of the population, population structure, relatedness in the population and minor allele frequency (MAF) are the crucial factors to map QTL (Fig. 5.1). LD is the nonrandom association of alleles/markers at different loci in a population that varies in self- and cross-pollinated crops. Several factors that affect Hardy-Weinberg equilibrium generate LD in the population. The extent of LD that exists in a population around a particular locus decides the resolution of mapping QTL.

The success of GWAS in the recent years can be attributed to advances in high-throughput genotyping technologies and development of well-sampled association panels. The identification of genome-wide markers particularly SNPs and INDELs facilitated the capture of LD between marker and the QTL. The mapping population (association panels) in GWAS can be a collection of wild species, landraces and cultivars from multiple breeding programmes, geographical distinct regions or

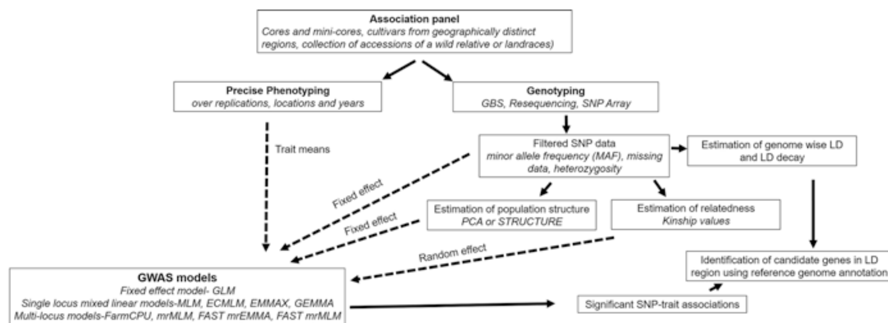

Fig. 5.1 A workflow of Genome wide association studies (GWAS)

regional breeding programmes that are amenable to repeated phenotyping. The development of densely genotyped association panels such as 2K SNP panel (McCouch et al. 2016) and 3K SNP panel (The 3000 Rice Genome Project, 2014) in rice are the best examples, tapping global diversity in a population. The well-developed cores and mini-cores in different crop plants could also be ideal populations for GWAS.

Extensive literature is available online that used GWAS for identification of QTL for several important traits. As an example, hundreds of publications have used this approach for mapping several traits in rice (Zhang et al. 2016) and maize (Xiao et al. 2017). It has been used in mapping of yield component traits in wheat (Neumann et al. 2011), *Fusarium* head blight resistance in barley (Massman et al. 2011), oleic acid content and fibre quality traits in cotton (Li et al. 2018), etc.

Mixed linear models (MLM) have been widely recommended for GWAS analyses which account for confounding effect of population structure (Zhang et al. 2005; Yu et al. 2006). A generalized MLM for GWAS analysis is:

$Y = X\beta + Zu + e$, where Y is vector of phenotypic observation for a particular trait of interest, X and Z are design matrices of fixed and random effects, β is a vector of fixed effect, u is a vector of random effect and e is unobserved vector of residuals and is normally distributed with mean zero and variance $\sigma^2 e$. Bonferroni correction is generally used to define significance of marker associated with the trait of interest and to control false-positive associations of SNP. Bonferroni correction is calculated simply by "α/number of SNP markers used for GWAS". The alpha (α) value could be 0.05 or 0.01 or lower depending upon stringency. The number of SNP markers is basically the number of independent tests. The choice of appropriate significant threshold is highly critical in GWAS (Hayes 2013). Since Bonferroni correction is a stringent measure of significant threshold, a number of other measures of estimating significant threshold have been suggested (Zhang et al. 2019). Because SNP markers are correlated and do not truly represent independent tests, so LD-based measurement of significant threshold could be more appropriate (Zhang et al. 2015). In another method, the total number of SNP markers is replaced by effective number of independent markers (Li et al. 2012).

A series of MLM-based methods have been developed for genetic dissection of complex traits which incorporates population structure as fixed effect and kinship estimates as random effect in the model. These models are either single-locus models such as MLM, ECMLM, EMMAX, GEMMA, etc. or multi-locus models such as FarmCPU, mrMLM, FAST mrEMMA, FAST mrMLM, etc (Zhang et al. 2019). Multi-locus models are being recommended to better control false-positive rates.

Though GWAS has been a widely recognized technique in the current phase, it suffers from several limitations such as power to detect meaningful associations that depend upon sample size, allele effect size and frequency, genetic heterogeneity and confounding effect of the background (Korte and Farlow 2013). In addition, deciding the appropriate significant threshold is critical to balance between false-positive SNP and significant associations. Currently, GWAS models do not incorporate the variation due to interaction between genes and environment which makes the process computationally intensive and time-consuming, but might include significant part of the variation. Further in most of the cases, outcomes of GWAS studies are not validated, limiting their practical utility. However keeping in view the emerging trends in better computational algorithms, decreasing genotyping costs and awareness of precise phenotyping results, GWAS could be a potential technique in the future for understanding genetic architecture of quantitativetraits.

5.3.3.2 NGS-Based Bulked Segregant Analysis

Mapping QTL in a large-size population is time-consuming, laborious and economically inefficient. Bulked segregant analysis (BSA) was suggested as a shortcut to identify linkage of molecular marker with the causal phenotype and has been used widely to map major-effect locus. In this technique, DNA of individuals showing extreme phenotypes in a segregating biparental population (usually F_2) are bulked and genotyped along with parents with a dense set of molecular markers. Any marker is found to be associated with trait of interest if it shows the same allele in the bulk and parent of similar phenotype (Michelmore et al. 1991). In the recent past, several BSA-based modifications have been developed to locate major-effect QTL governing quantitative traits. These modifications are based on whole genome resequencing bulks of extended tails in a large population, resulting in reduced genotyping cost, saving time and comparable statistical power of entire population analysis (Zhou et al. 2016; Nguyen et al. 2018).

QTL-seq is one such approach for rapid identification of plant QTL in a biparental population (Takagi et al. 2013a). In this technique, the segregating mapping populations derived from a cross between two contrasting parents are evaluated for the target traits preferably in replications and multi-environments. Two DNA bulks (high and low bulk) of 10–20 individuals showing extreme trait values are sequenced on suitable NGS platform. The NGS short reads of each bulk are aligned to the reference genome to identify genome-wide SNPs and SNP index is estimated. The SNP index is frequency of alleles in NGS reads aligned at a genomic position. The SNP index will range from 0 to 1 with 0.5 at most of the genomic positions. The

SNP index of "1" in high bulk and "0" in low bulk at a particular position will be the putative genomic region harbouring the QTL. The approach has been used to identify QTL for important traits in several crop plants (Table 5.1).

MutMap approaches can be used to uncover major mutations governing target phenotype. MutMap was suggested as a method to locate QTL using F_2 population

Table 5.1 Mapping of major-effect QTL and candidate genes for quantitative traits using QTL-seq approach

Crop	Trait	Gene/QTL identified	References
Rice	Cold tolerance	*qCTSS-1, qCTSS-2b, qCTSS-8*	Yang et al. (2013)
	Blast disease and seedling vigour	*qPHS3-2*	Takagi et al. (2013a)
	Salt tolerance	OsRR22 Saltol	Takagi et al. (2015) Tiwari et al. (2016)
	Nitrogen use efficiency	*qNUE6*	Yang et al. (2017)
	Grain length and weight	*qTGW5.3*	Yaobin et al. (2018)
	Grain weight	*qTGW3.2,qTGW3.1, qTGW3.3*	Xu et al. (2015a)
	Dwarfness	*asd1*	Kadambari et al. (2018)
	Resistance to root knot nematode	Candidate genes on chromosome 11	Lahari et al. (2019)
	Resistance to BPH	*QBPH4.1, QBPH4.2*	Kamolsukyeunyong et al. (2019)
	Cooked grain elongation	*qGE4.1, qGE6.1, qGE6.2*	Arikit et al. (2019)
Cabbage	Early flowering	*Ef2.1*	Shu et al. (2018)
Groundnut	Rust and late leaf spot resistance	Candidate genes, Aradu.H1HIG, Aradu.7MV8U	Pandey et al. (2016)
Soybean	Plant height	*qPH13.1*	Zhang et al. (2018)
Chickpea	Pod number	Genomic regions on chromosome 4 (Caq(a)PN4.1: 867.8 kb, Caq(a) PN4.2: 1.8 Mb)	Das et al. (2016)
	Flowering time	*CaqDTF4.1, CaqDTF4.2*	Srivastava et al. (2017)
Foxtail millet	Heading date	*qDTH2, qDTH7*	Yoshitsu et al. (2017)
Tomato	Early flowering	*EF1*	Ruangrak et al. (2018)
	Early fruit ripening	*ER fruit*	Ruangrak et al. (2019)
Cucumber	Early flowering	*EF1.1*	Lu et al. (2014)
	Flesh thickness	*fft2.1*	Xu et al. (2015b)
	Fruit length	*fl3.2*	Wei et al. (2016)
	Subgynoecy	*sg1.1, sg1.2, sg3.1*	Win et al. (2019)
Watermelon	*Fusarium oxysporum* f. sp. *niveum*	*Fo-1.1*	Branham et al. (2018)

derived from a cross of homozygous recessive mutant and wild type (Abe et al. 2012). The DNA of ≥20 F_2 individuals showing mutant phenotype is bulked and sequenced along with wild type with >10X coverage. The SNP index is calculated by aligning reads to wild-type genome as the reference. The SNP index of "1" or near to "1" will be the causal genomic region governing the trait variation. MutMap has been used to identify functional mutation responsible for salt tolerance in ethyl methanesulphonate (EMS) based mutant of rice cv. Hitomebore (Takagi et al. 2015).

MutMap will be inefficient to identify causal mutation, if the wild-type reference genome has gaps at the position of causal mutation. Therefore, de novo assembly of wild-type genome is used in MutMap-Gap (Takagi et al. 2013b). The technique enabled the identification of blast resistant gene *Pii* in rice variety Hitomebore which was absent in Nipponbare reference genome (Takagi et al. 2013b).

MutMap+ is another version of Mutmap which can identify the causal mutation without crossing mutant with wild-type plant (Feikh et al. 2013). The approach saves time used in getting homozygous mutant and developing segregating population. In this approach, M_2 individual heterozygous for causal mutation is self-pollinated to generate M_3progenies which are expected to segregate in 1:3, if controlled by single major mutation. Similar to QTL-seq, two bulks (showing mutant and wild-type phenotype) are sequenced and SNP index is calculated.

The cost of whole genome DNA resequencing will increase in the species with larger genome size and having more repetitive DNA. In that case, BSR-seq can be a suitable strategy which identifies eQTL (expression QTL) region and generates data of gene expression at all the genomic loci (Liu et al. 2012). The differential expression of genes in two bulks can be used to identify candidate genes responsible for mutant phenotype. Unlike RNA-seq experiments, BSR-seq can also use unreplicated data to map QTL. Even the kind of tissue for RNA-seq analysis in BSR-seq does not impact much the mapping result, but will affect the results of differentially expressed genes. Because even if causal candidate gene expression is absent in the sample tissue used for RNA-seq analysis, the SNPs linked with causal candidate gene that are expressed in RNA-seq analysis will be used as markers to map the causal gene. However for dual advantage of mapping and identifying differentially expressed candidate genes, it would be ideal to use the tissue, in which candidate gene is expressed for RNA-seq analysis (Liu et al. 2012). BSR-seq has been successfully used for mapping of stripe rust-resistant loci *YrMM58* and *YrHY1* on chromosome 2AS (Wang et al. 2018) and leaf senescence gene *els1* on chromosome 2BS (Li et al. 2018) in segregating biparental population in wheat.

5.4 Genomic Selection

The prime emphasis of any plant breeding programme is to develop cultivars with higher genetic values that depend upon selection of desirable combination of alleles in a population. The efficiency of plant breeding programme can be measured in

terms of genetic gains over time which can largely be increased by selection accuracy and reducing the generation interval.

$$\text{Genetic gains over time } (\Delta G) = \frac{ir\sigma_{\text{A}}}{Y}$$

(*The numerator of equation has selection intensity (i), selection accuracy (r) and additive genetic variance (*σ_{A}*). The denominator of the equation is generation time (Y) which is years per cycle. From all these factors, plant breeder can focus on increasing "r" and decreasing "Y".*)

Since quantitative traits are affected by the environment, the accuracy of selection will depend upon precise phenotyping procedures that involve replications and multi-location and multi-environment evaluation. However the cost of phenotyping increases with the number of lines to be evaluated and the number of test locations and environments. Since 1980, availability of DNA-based molecular marker with their direct associations with important traits facilitated marker-assisted selection (MAS), thus saving time, energy and cost of field-based rigorous evaluations. MAS became an important tool for selection of traits with low heritability, combining multiple genes and avoiding transfer of undesirable genes and early generation selection. However this method is effective for few genes of large effect, but not for quantitative traits, and still required rigorous field selection. In addition, MAS requires marker information from initial investment on high-resolution mapping and cloning. Most of the present advances in mapping and cloning lack confidence to capture minor-effect QTL which are equally important in any crop improvement programme.

Meuwissen et al. (2001) proposed genomic selection (GS), a form of marker-assisted selection which is more of a breeding tool than a research tool. GS uses phenotypic and genotypic data from the past trials to predict value of individuals that have not been phenotyped. It uses data from entire genome and information of relatives to predict genomic estimated breeding value (GEBV) of each individual. GS ignores the time-consuming steps of QTL mapping and cloning and takes care of both major-effect and minor-effect loci.

Basically, GS uses genotypic and phenotypic data to estimate allele effects or GEBV at all the loci. Essentially training population is high-throughput genotyped and phenotyped (Lorenz et al. 2011). GEBV of all loci is used to select best progenies from selection population which is only genotyped. The selection population can be generated from crossing best progenies based on GEBV from training population or could be a part of population from which training population is derived. Selection population is generated every year, and procedure of genomic selection is repeated to get elite lines that can enter yield trails. Several GS models such as ridge regression best linear unbiased predictors (rrBLUP), least absolute shrinkage and selection operator (LASSO), Bayesian models, etc. are used to calculate GEBV. These models takes care of large p and small n problem, in which more numbers of effects (p) are to be estimated than less number of observations (n) (Lorenz et al. 2011).

A number of factors impact the effectiveness of GS including size of training population, population structure, correlation between training and selection population, number of markers and model selection, complexity of trait and phenotyping of training population. The training population is derived from breeding populations or biparental populations and must have phenotypic and genotypic variation. Where size of training population is a trade-off between cost and accuracy, limited population structure increases correlation between training and selection population thus accuracy. In addition, accurate phenotyping of training population is essential to get accurate GEBV for further selections.

Keeping in view the challenges posed by changing environment conditions and advances in genotyping and phenotyping, GS could become a future breeding tool for combining variability for complex traits in elite genotypes.

5.5 Future Prospects

Advances in quantitative genetics have utmost potential to confront the current and future global challenges primarily imposed by changing climate and burgeoning population. But it will be possible with utilization of these advances at wider scale so that acceleration in crop improvement programmes could be gained at global as well as regional level. As an example, due to the higher cost involved in establishing advanced phenotyping laboratories, their applications are still limited to few institutes particularly in the developed countries. Therefore, there is a need to develop such advances which are economical besides high-throughput. However in the current scenario, emphasis may be given to create more funding opportunities to establish such type of facilities at staggered and common places to increase accessibility to most of the institutes. Due to the pace of advancements, lack of trained manpower is also a major concern. Training hubs may be established which is responsible for training young researchers and students in judicious use of advances in quantitative genetics.

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Chapter 6
Haploid Production Technology: Fasten Wheat Breeding to Meet Future Food Security

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Abbreviation

CIMMYT International Maize and Wheat Improvement Center
DH Doubled haploid

6.1 Introduction

Bread wheat (*Triticum aestivum* L.) is one of the most important crops in the world and grows in more countries than rice (*Oryza sativa* L.) and maize (*Zea mays* L.). It not only provides a source of calories, but it is also the number one source of protein in many countries (Hawkesford et al. 2013). Doubling food production (from 2005 to 2050) may be necessary to match increasing food demands in the future, as the world population is still increasing until 2050 and life style changes such as urbanization and an expanding middle class further exacerbate the potential for food shortages (Godfray et al. 2010; Tilman et al. 2011). However, this is not an easy task, because yield increase in wheat and other cereals is declining in some parts of the world (Finger 2010; Brisson et al. 2010). In addition to this, many reports predict that climate change will significantly decrease global food (Schmidhuber and Tubiello 2007) and the damage from climate change will be greatly intensified in the near future (Lobell and Tebaldi 2014). To prepare for a climate-changing world, wheat varieties must have a sufficient level of drought and heat tolerance to avoid significant reductions in global wheat production. Such a scenario could create political instabilities in some countries, as mentioned by Kelley et al. (2015), who pointed out that Syria's civil war would be a consequence of a prolonged 3-year

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drought. Emerging disease has threatened wheat production for the last two decades. When Ug99 stem rust appeared, more than 70% of wheat varieties in the world did not have resistance (Singh et al. 2015). More recently, wheat blast jumped from Brazil to Bangladesh, creating fears that the disease might spread to India because wheat varieties in Bangladesh and India did not have resistance (Malaker et al. 2016; Mottaleb et al. 2018). These diseases have highlighted the need of wheat breeding programs to react swiftly to urgent threats.

To cope with these difficulties, it is obvious that we need to find better and more efficient breeding strategies to produce improved wheat varieties in less time. One area for the improvement is the reduction of breeding time. Wheat breeding takes several years to develop a new variety from F_1 to have F_4, F_5, or more advanced generations that are genetically homologous. If we can go directly from F_1 to a homologous condition, skipping F_2–F_3 or F_4 segregating generations, it can save years of breeding time, which would lead to a faster release of new variety and bring a higher genetic gain or improvement in the same period. Researchers have known that doubled haploid (DH) is one such technology. Wheat DH methods have significantly advanced in the last several decades, and DH wheat breeding and DH-derived varieties are already common in the Western countries, where it has become more important to meet future food security.

6.2 Haploid Plant Formation

The first report of finding a haploid plant can date back to 1922 in jimson weed, *Datura stramonium* L. (Blakeslee et al. 1922). Ten years later, Kihara and Katayama (1932) reported the first finding of natural haploid *Triticum monococcum* L. (diploid ancestor species of bread wheat). The frequencies of a naturally occurring haploid plant are very rare, as it was reported about 0.01% or 0.5% in *T. monococcum* (Katayama 1934; Smith 1946) and 0.1% in maize (Chase 1949). It is, therefore, essential to artificially induce haploid for practical uses. The induction methods can be classified into two categories: in situ and in vitro. The in situ methods are modified pollination techniques, while the in vitro methods are based on cultures of gamete cells (Gilles et al. 2017b). Soon after the first report of plant haploid in 1922, a number of in situ methods were discovered to produce artificial haploid plants, which includes x-ray treatment on pollen (Blakeslee et al. 1922; Stadler 1931; Kihara and Katayama 1932; Ivanov 1938), wide crossing/interspecific crosses (Goodspeed and Avery 1929; Gerassimova 1936; Badenheizen 1941), high- and low-temperature treatments (Clausen and Mann 1924; Chizaki 1933; Povolochko 1937; Nordenskiold 1939), use of abortive pollen (Nakamura 1933; Webber 1933), delayed pollination (Kihara 1940; Smith 1946; Seaney 1955), and chemicals (Smith 1943; Levan 1945; Deanon 1957). An interspecific haploid inducer line was also found in a maize inbred line "stock 6" whose pollen induced about 2% haploid formation (Coe 1959).

In wheat, wide crossing was the first case of a haploid plant finding, which was derived from crosses *T. compactum* Host × *Aegilops cylindrica* L. (Gaines and Aase 1926). The effect of x-ray on the formation was intensively studied in the 1930–1940s

using *T. monococcum* and tetraploid wheat (Kihara and Katayama 1932; Katayama 1934; Yefeikin and Vasiljev 1935; Kihara and Yamashita 1939). The induction rates by x-ray treatment on pollens in *T. monococcum* were reported up to 17% (Katayama 1934) or 2% (Smith 1946). However, one problem of x-ray treatments is that it does not always result in the production of haploids (Smith 1946). Chizaki (1933) reported that a high temperature could induce haploid plant in *T. monococcum*, and Kihara (1940) showed that a delayed pollination (12 days after emasculation) caused 20% haploid production in *T. monococcum*. Cytoplasmic substitution can also induce haploid; Kihara and Tsunewaki (1962) reported up to 15% haploid induction in a *T. aestivum* line (cytoplasm substituted by *Aegilops caudata* L.) and triticale line (cytoplasm substituted by *Ae. caudata*).

In vitro methods occurred later in the 1960s. Cultured anthers of *Datura inoxia* Mill that developed into haploid plants were first demonstrated in 1964 (Guha and Maheshwari 1964). Soon after these reports, anther culture was also achieved in wheat (Ouyang et al. 1973; Picard and De Buyser 1973; Liang et al. 1987). Microspore culture was then developed as the use of microspores themselves instead of anthers (Datta and Wenzel 1987; Hu et al. 1995; Touraev et al. 1996; Hu and Kasha 1997) as the two methods are induction of microspore cells.

Other cultures such as microspore culture and maize methods are also common in wheat doubled haploid production. Even though the former two in vitro methods can produce a larger number of DH plants from a spike, they are highly genotype dependent and do not apply to all wheat lines. Maize in situ method, on the other hand, produces less DH plants from one spike, but it is much less genotype independent and as such applicable to all wheat genotypes.

6.3 Maize Method and Wide Crossing

It has been well known since the 1920s, that many interspecific crosses among Triticeae species can induce haploid formation (for review, see Ishii et al. 2016). For wheat, examples have been reported in *T. compactum* × *Aegilops cylindrica* (Gaines and Aase 1926), in *T. durum* Desf. × *T. monococcum* (Vasiyev 1936), *T. turgidum* L. × rye [*Secale cereale* L.] (Nakajima 1935), *T. aestivum* × barley [*Hordeum vulgare* L.] (Koba et al. 1991), *T. aestivum* × *H. bulbosum* L. (Barclay 1975; Pickering and Morgan 1985), and *T. aestivum* × *Ae. caudata* (by the authors; data not shown).

6.3.1 Haploid Induction with **H. bulbosum** *and Panicoideae Species*

The first practical doubled haploid production system in wheat is the *Bulbosum* method in which wheat (female parent) is crossed with pollen of *H. bulbosum*. The *Bulbosum* method was originally developed in cultivated barley (*H. vulgare*). When

barley was crossed with *H. bulbosum*, all *H. bulbosum* chromosomes were eliminated, leaving barley haploid (Kasha and Kao 1970). The same phenomenon happened with wheat, when wheat was used as female parents (Barclay 1975; Pickering and Morgan 1985), showing the haploid induction rate is more than 50% in some wheat genotypes (Barclay 1975; Inagaki and Snape 1982). The main problem of this method is that wheat has crossability genes that control the crossability of wheat with different plant species including rye, *H. bulbosum*, and *Aegilops*. The crossability of wheat is controlled by two major genes, *Kr1* and *Kr2* (Lein 1945), affecting pollen tube growth (Riley and Chapman 1967; Snape et al. 1980) and one suppressor gene of *Skr1* (Tixier et al. 1998). In the presence of a dominant alley of *Kr1* and *Kr2*, wheat will show 0–10% crossability with wild relative species (Lein 1945), and most Western wheat lines have a dominant alley of *Kr1* and *Kr2* genes (Zeven 1987). Snape et al. (1979) showed that 16 out of 17 wheat lines had 0% crossability with *H. bulbosum*, except for one Chinese landrace. To overcome this problem, alternative pollen sources that are genotype independent of the crossability genes have been identified, including maize (Laurie and Bennett 1987; Inagaki and Tahir 1990), teosinte [*Zea mays* L. spp. *mexican*; wild maize species] (Ushiyama et al. 1991; Suenaga et al. 1998), eastern gamagrass [*Tripsacum dactyloides* (L.) L.; maize wild relatives] (Riera-Lizarazu and Mujeeb-Kazi 1993), sorghum [*Sorghum bicolor* (L.) Moench] (Laurie and Bennett 1986), pearl millet [*Pennisetum glaucum* (L.) R. Br.] (Ahmad and Comeau 1990), Job's tears [*Coix lacryma-jobi*] (Mochida and Tsujimoto 2001), and cogongrass [*Imperata cylindrical* (L.) P. Beauv.] (Chaudhary et al. 2005). The authors have also confirmed that maiden silvergrass [*Miscanthus sinensis*] can induce wheat haploid formation (data not shown). One of the interesting things about these species is that they all belong to the subfamily Panicoideae in the grass family (Poaceae) (Kellogg 2001). Among those inducer species, maize has been used most for wheat doubled haploid production, maybe due to a larger embryo size (Inagaki and Mujeeb-Kazi 1995), as well as being easier to access and handle than other species. Yet it should be noted that the regeneration rates of green plants were higher in pearl millet and sorghum crosses than maize crosses in some reports (Inagaki and Mujeeb-Kazi 1995). The induction rate of *I. cylindrica* L. is also similar or higher than maize by up to 75% (embryos/pollinated florets) (Chaudhary et al. 2005). Even though this is in wild species, one advantage of this species is the similar flowering time to wheat (Chaudhary et al. 2005).

6.3.2 Maize Method and Pollination

The procedure of the maize method is summarized in Fig. 6.1. In wheat × maize crosses, it will not develop grains and endosperm, so it is necessary to stimulate initial grain development, followed by embryo rescue and chromosome doubling. For the pollination, the condition of both wheat stigmas and maize pollens is important. Laurie (1989) reported intact/closed glumes showed the highest frequency (56% of pollinated florets) and not cut glumes (exposed stigmas). To improve the

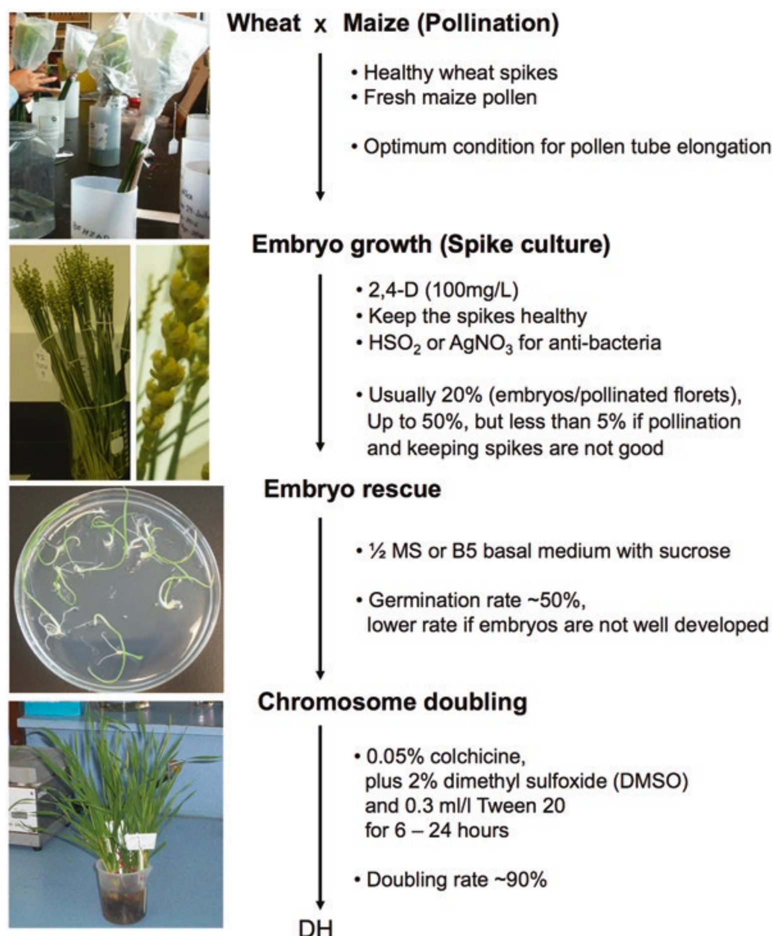

Fig. 6.1 The procedure of maize-based doubled haploid production

frequency in cut glumes, plastic bags are commonly used to keep moisture inside and as such stigmas healthy. For maize pollens, both temperature and light intensity as well as seasons can affect the efficiency of embryo formation, as it influences pollen tube growth (Campbell et al. 2001), as well as wheat and maize plant conditions. Campbell et al. (1998) demonstrated light intensity at 1000 μmol/m^2/s irradiance at 22/17 °C (day/night) for the greatest recovery of embryos across different maize genotypes. The success rate in wheat × maize in terms of embryos/pollinated florets has been reported mostly around 20% (Inagaki and Tahir 1990; Inagaki and Mujeeb-Kazi 1995; Ushiyama et al. 2007; Hussain et al. 2012) and up to 40–50% (Laurie 1989; Inagaki and Tahir 1990; Suenaga et al. 1997, 1998), but it was sometimes 10% (Xynias et al. 2014) and even to 0% in our experiences (data not shown). Even though it is less affected by the genotype of wheat and the presence of *Kr1* and *Kr2* genes, embryo formation rate is still influenced by these factors

to a certain extent (Inagaki and Mujeeb-Kazi 1995). Wheat genotypes with high crossabilities (e.g., CS and Norin 61) tend to have more embryo formation rates (Suenaga and Nakajima 1989; Inagaki and Tahir 1990; Inagaki and Mujeeb-Kazi 1995; Hussain et al. 2012). Maize lines also had different induction capacities for embryo setting and wheat haploid plant (Inagaki and Tahir 1990; Ushiyama et al. 1991), and teosinte (the maize ancestor) showed the highest and the most stable induction rate in the report of Ushiyama et al. (1991).

6.3.3 Chemical Stimulation for Grain Swelling

The stimulation of grain forming/swelling can be done by chemical treatments. The solution will be applied by spraying or dropping it to florets, injecting it into the stem of spikes, or culturing detached spikes. The two forms of spike culture methods are employed for the treatment: cut or detached spikes or on-pant spikes. Inagaki (1997) compared two methods and concluded that the detached spike method was more efficient for embryo and haploid plant recovery than on-pant spike method, in addition to the convenience of handling spikes in laboratory. Yet the opposite results were reported in bread wheat (Laurie 1989). It is also true that careless handlings of spikes in detached spike culture will easily lead to serious damages on embryos and, thus, produce a very low embryo setting (the authors' unpublished data). The detached spike, while a powerful method, should only be employed with skilled personnel.

Spike culture is the most commonly used method in the solution of 2,4-D (100 mg/L) just after pollination (Inagaki and Mujeeb-Kazi 1995). Ushiyama et al. (2007) suggested 2,4-D (50–100 mg/L) as optimum for haploid wheat production using maize, because they observed that a concentration higher than 100 mg/L inhibited embryo development. A mixture of 2,4-D (50 mg/L) and gibberellic acid (GA3) (100 mg/L) may be alternative for the injection or spraying (Pienaar et al. 1997; Singh et al. 2001; the authors' unpublished data). Dicamba (3,6-dichloro-*o*-anisic acid) or ZEN (zearalenone) induced a higher embryo setting, but this resulted in a lower haploid plant yield than 2,4-D (Pienaar et al. 1997; Biesaga-Koscielniak et al. 2003; García-Llamas et al. 2004). The optimum concentration of 2,4-D for durum is different from bread wheat. Niu et al. (2014) reported that the most effective way for durum is to spray for pollinated spikes with 2,4-D solution (213.05 mg/L, pH = 10.36).

With the spike culture in the solution, it is important to keep the solution clean and the spikes healthy. The nutrition-rich medium provides a good condition for bacteria expansion, which chokes the spikes preventing the movement of water and nutrition to embryos. To avoid this problem, it is necessary to change the solution every 1–2 days and inhibit the bacterial growth by adding acids such as sulfurous acid (8 mL/L = 6% HSO_2) to the solution (Inagaki 1997; Hussain et al. 2012). $AgNO_3$ is also effective as antimicrobial (Bokore et al. 2017), and the addition of $AgNO_3$ together with 2,4-D gave a good yield of embryos in a durum haploid production report (Almouslem et al. 1998; Ayed et al. 2011a; Bokore et al. 2017).

6.3.4 Embryo Rescue

Around 2–3 weeks after pollination, grain or caryopsis reaches its life span and cannot sustain an embryo inside any longer; thus it is necessary to take out the embryo and place it into a culture medium. The common mediums are full-strength MS (Murashige and Skoog 1962) or ½ MS or B5 basal medium (Gamborg et al. 1968) with sucrose and additional elements (Inagaki and Mujeeb-Kazi 1995; Ayed et al. 2011b). In comparison, ½ MS and B5 medium were more efficient than MS (Cherkaoui et al. 2000; Xynias et al. 2014). For durum wheat, B5 medium resulted in more germination than ½ MS medium (Ayed et al. 2011b). The commonly used concentration of sucrose is 20–40 g/L.

After the rescue, embryos will be incubated for weeks. Incubation conditions are recommended to be at 20–25 °C with a 16 h day, as there is flexibility as the temperature rises. The germination rate of embryos has been reported 30–60% in many publications (Inagaki 1997; Ushiyama et al. 2007; Hussain et al. 2012). One germination rate factor is the size of the embryo. If the embryo size is very small (= not developed well), it may not germinate. For wheat × maize, an embryo size of less than 1.5 mm may not germinate well (Almouslem et al. 1998). The nurse endosperm method improves the germination rate, in which the excised embryos were placed on the 20-day-old seed endosperm prior to culturing in the MS medium or B5 medium (Niu et al. 2014).

6.3.5 Chromosome Doubling

Spontaneous chromosome doubling rate in nature occurs at very low frequency, and so it is necessary to make artificial chromosome doubling of haploid plants. The most common method is the treatment of seedling stage plants with a colchicine solution. The chemical affects spindle fiber formation during metaphase of mitosis, resulting in chromosome doubling in some cells. The treatment can be usually done by dipping the plants (including apical meristems) into colchicine solution at concentration of 0.05–0.1% plus 2% dimethyl sulfoxide (DMSO) and 0.3 mL/L Tween 20 for 6–24 h (Jensen 1974; Thiebaut et al. 1979; Inagaki 1985). A higher concentration of colchicine applied for a longer time is generally more effective for chromosome doubling; however, it is important to note that the toxicity of colchicine may kill the plants. If the plants are growing properly before and after the treatment, the success rate of seed setting is more than 95% (Inagaki 1985). Since colchicine is carcinogenic, caffeine (3% concentration for 24 h of treatment) can be used as alternative chemical substances. But the efficiency for the doubling was less than colchicine (Thomas et al. 1997).

A completely different chromosome doubling method is to use nitrous oxide (N_2O) gas just 24 h after the pollination. The gas treatments under a high pressure (3 atm.) for 15 h showed a chromosome doubling rate of more than 70% of (Kihara and Tsunewaki 1960). Hansen et al. (1988) also reported that the induction rate with

N_2O gas (6 atm. for 24–48 h) was equivalent with colchicine treatment (0.01% and 0.005% for 24 h) without any damages on plants, which colchicine treatment usually had. Another benefit of this gas treatment is that the chromosome doubling can be applied in one or two cell stages of fertilized eggs that result in chromosome doubling of the whole plant instead of chimeric doubling by colchicine treatment. The consequence will be a much higher seed production from the N_2O-treated plants, as well as being able to forgo an extra round of seed multiplication for field tests.

6.4 Anther Culture and Microspore Culture

Anther culture and microspore culture methods are techniques referred to as induced androgenesis by which immature pollens/microspores switch their cell fate to callus and pseudoembryos (embryo-like structure) or embryoids instead of mature pollens (Reynolds 1997). The differences between anther and microspore culture mainly lie in the use of anthers or isolated microspores. It can potentially produce a larger amount of DH than the maize method, as an anther contains thousands of pollens. Although the first report of anther culture in wheat was in the 1970s (Chu et al. 1973; Ouyang et al. 1973; Craig 1974), it was not practical to use wheat doubled haploid production for decades due to problems of low yield of callus or embryoids (Chuang et al. 1978) by the use of solid medium (Zhou and Konzak 1989; Zheng 2003) and high frequency of albino plants over green plants (Ouyang et al. 1973; Zheng 2003). Later, the use of a liquid medium improved the ratio of callus or embryoids (Chuang et al. 1978; Zhou and Konzak 1989). Anther culture and microspore culture consist of three steps: (1) pretreatment of microspore donor plants, (2) induction of embryoids, and (3) regeneration of the plant (Fig. 6.2).

6.4.1 Pretreatment

It is the process to change the cell fate of immature pollens to embryogenesis instead of pollen grains. Immature pollens in different stages can have different effects for embryogenesis. Mid- to late-uninucleate stage cells are the most sensitive for the induction, so it is crucial to maximize the use of cells at these stages (Haggag and El-Hennawy 1996). It is also crucial to keep donor plants in good growth conditions (Orshinsky and Sadasivaiah 1997). Any kind of stress can induce the change of cell fate, including disease, starvation, cold/heat stresses, and chemicals (Zheng 2003). In wheat, cold treatment is usually applied at 4–7 °C for 10–12 days (Gustafson et al. 1995; Ingram et al. 2000; Redha and Talaat 2008; Lantos et al. 2018). Alternatively, sugar starvation (such as 0.3 M mannitol or 0.25 M maltose) can be used as a pretreatment (Mezja et al. 1993; Hu et al. 1995; Zheng et al. 2001; Castillo et al. 2015). Higher temperature at 33 °C for 2–3 days is also very effective for embryoid induction (Touraev et al. 1996, 1997; Lantos et al. 2018). More recently,

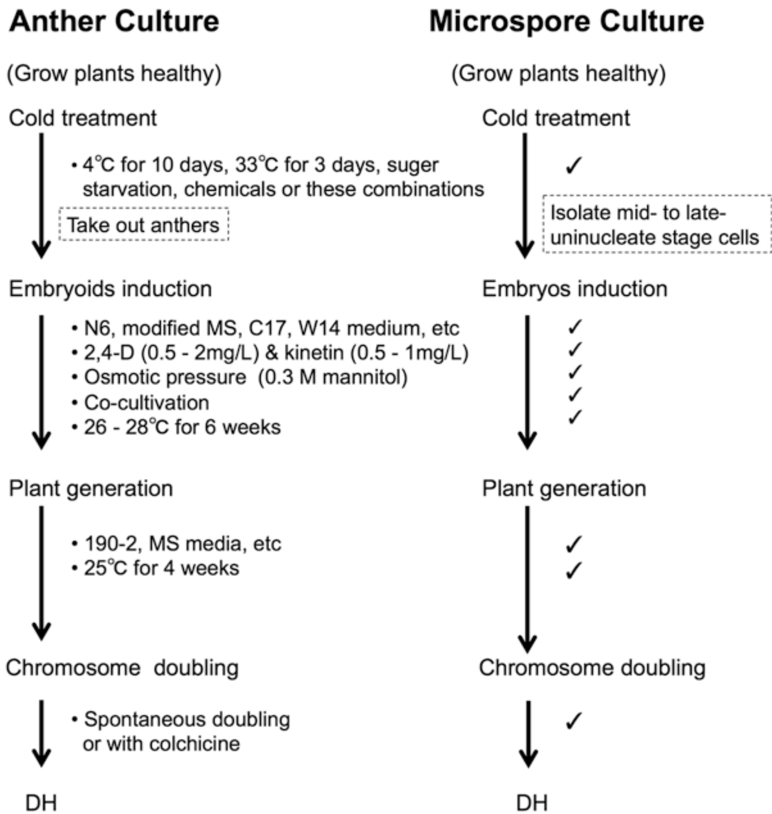

Fig. 6.2 The procedure of anther and microspore culture-based doubled haploid production. ✓ = same as the left

some chemicals have been known to have similar effects, such as colchicine (Redha et al. 1998), 2-hydroxynicotinic acid (2-HNA), 6-benzylaminopurine (BAP), 2,4-D (Zheng et al. 2001; Liu et al. 2002), and n-butanol (Soriano et al. 2008; Broughton 2011). Different treatments have additive effects, so the combination of treatments is becoming more common to achieve a higher efficiency of DH production (Santra et al. 2012; Lantos et al. 2018).

6.4.2 *Induction*

After the pretreatment, callus or embryoid formation will be induced by placing anthers or isolated microspores into an induction medium. In the microspore method, it requires one extra step of isolation and purification of microspores that can provide a more homogeneous population of mid- to late-uninucleate stage cells, through filtration by gradient centrifugation (Mezja et al. 1993).

It is important to provide both optimum physical conditions and nutrition and plant growth hormones. The physical conditions include temperature, osmolarity, and solidness of medium. Optimum incubation temperature is 26–28 °C in dark, and osmolarity is 300–320 mOsmol/kg H_2O, which can be commonly provided with maltose (0.25 M) or mannitol (0.3 M) (Zheng 2003). Liquid medium instead of solid medium improves callus induction significantly (Zhou and Konzak 1989; Zhou et al. 1992). The commonly used basal media are derivative of N6 (Chu 1978), Potato-2 (Chuang et al. 1978), modified MS (Hu et al. 1995; El-Hennawy et al. 2011; Castillo et al. 2015), C17 (Wang and Chen 1983; Weigt et al. 2016), and W14 (Ouyang et al. 1989; Lantos et al. 2016, 2018). Plant growth hormones are essential for induction, (Zheng 2003), and a mixture of 2,4-D (0.5–2 mg/L) and kinetin (0.5–1 mg/L) is most commonly used for this purpose (Weigt et al. 2016; Lantos et al. 2018). The source of carbohydrate is maltose or sucrose; however, maltose may be better, as sucrose changes osmolarity in mediums (Hunter 1988; Zhou et al. 1991; Mezja et al. 1993). It also includes Ficoll when it is prepared in a liquid medium instead of a solid medium (Zhou and Konzak 1989; Redha and Talaat 2008; Castillo et al. 2015; Lantos et al. 2018). Co-cultivation with nurse cells, such as live ovaries or ovary conditioned medium, (OVCM) increases the induction rate, which is more prominent for less responsive genotypes (Mezja et al. 1993; Puolimatka et al. 1996; Hu and Kasha 1997; Puolimatka and Pauk 1999; Zheng et al. 2002). Castillo et al. (2015) reported up to 6- and 11-fold increase in the numbers of embryos and green plants, respectively, having a greater effect on a medium-low responding cultivar and ovary genotype; the developmental stage significantly affected microspore embryogenesis.

6.4.3 Regeneration

Once embryoids form and reach around 2 mm in size, they will be transferred onto semisolid regeneration mediums to generate into green plants. The most commonly used mediums are 190-2 (Zhuang and Jia 1980) and MS (Zheng 2003; Santra et al. 2012; Weigt et al. 2016). The incubation should be under light, and embryoids will germinate on the media in a week. Once it germinates, it will follow the procedure of the maize method.

Albino plants usually appear which is still a limiting factor in anther/microspore culture (Andersen et al. 1987; Kumari et al. 2009; Torp and Andersen 2009). The frequency of albino plants depends on genotypes (El-Hennawy et al. 2011). Islam and Shahinul (2010) reported that the transfer of large and heart-shaped embryoids could reduce lower albinos in three genotypes. One pretreatment chemical, n-butanol, also has an effect on the green/albino plants' ratio (Broughton 2011). In anther culture, autonomous chromosome doubling was observed with relatively high frequency, more than 30% (Zheng et al. 2001; Lantos and Pauk 2016; Lantos et al. 2016), and as such, the chromosome doubling may not be necessary if it has a large number of embryos. Otherwise, the chromosomes will be doubled using colchicine, which is the same as in the maize method.

6.4.4 Anther Culture vs. Microspore Culture

When comparing the efficiency between anther culture and microspore culture, microspore has the potential to produce larger numbers in general, if the conditions are favorable. Liu et al. (2012) showed the production of up to 5000 green plants/one spike was possible in several wheat genotype. However, it has been also reported that albino frequency is generally higher in microspore than in anther culture. Lantos et al. (2018) compared the two methods using four spelt wheat genotypes and observed that the mean ratio of green plants/albino plants was 1:100 in microspore culture while of green plants/albino plants it was 2.5:1 in anther culture, concluding that microspore culture is not practical for spelt wheat while anther culture was efficient for doubled haploid production.

6.4.5 Anther/Microspore Culture vs. Maize Method

One obvious advantage of in vitro androgenesis is the number of DH plants that can be potentially developed from one spike. Many reports showed more than 80 green plants per spike in highly responding genotypes (Lie et al. 2012; Lantos et al. 2013, 2018), while it will be 4–20 embryos per spike (or 50% of one spike = 20–30 embryos at most) in the maize method (Laurie 1989; Inagaki and Tahir 1990; Suenaga et al. 1997, 1998). Yet the genotype-dependent nature of androgenesis is the most serious limitation to certain breeding programs (Holme et al. 1999). For low responding phenotypes, anther culture cannot promise any DH output. One strategy is to use a high responding line for crosses (Tuvesson et al. 2000). Another way to look at the two in situ and in vitro systems is that these two are compensative. For high responding genotypes, in vitro methods are the option to produce a large number of DHs (Andersen et al. 1988), and then the maize method can be used for the remaining low responding genotypes, which actually is a routine practice in some breeding programs in Japan (personal communication).

6.5 DH Practices in Wheat Breeding

Doubled haploid technology has been already applied for wheat breeding for years, especially in Western countries. The first wheat variety 'Jinghua #1' using anther culture technology was reported in the early 1980s in China (Hu et al. 1983). According to Thomas et al. (2003), 21 DH-derived wheat cultivars had been released in China, Germany, Brazil, Canada, France, Hungary, Sweden, and the UK by 2003. Canada had released 27 wheat varieties using DH technology from 1997 and 2010, and these varieties cover more than 1/3 of Canada's wheat field (DePauw et al. 2011a). An additional 14 varieties have also been released since 2011 (Table 6.1). In

Romania, four of eight bread wheat varieties were maize-based DH derived in 2002–2008. One wheat cultivar, 'Glossa,' covered about 16% of the country's wheat area (Saulescu et al. 2012). Recently, the USA has joined into DH wheat variety holders, registering three varieties, UI Sparrow, Langin, and Avery in 2018 (Chen et al. 2018; Haley et al. 2018a, b), even though some DH wheat varieties had already been available from the private sector such as SY 107 (https://agriprowheat.com/wheat-variety/pacific-northwest/sy-107), SY Ovation (https://agriprowheat.com/wheat-variety/pacific-northwest/sy-ovation-0), LCS Chrome, and LCS Drive (https://www.aosca.org/wp-content/uploads/2017/10/2016SG_Report_FINAL.pdf). The number of DH-derived wheat varieties in the USA may increase in the future, because in the last 10 years, at least two universities in the USA have opened DH facilities, one in Kansas State University (http://www.heartlandinnovations.com/our-programs/doubled-haploid-production) and another in Washington State University (http://css.wsu.edu/facilities/dhlab/). The three varieties released in 2015–2016 were developed in 2010, and the two DH facilities were open in 2012 and 2015. The Kansas facility can produce around 50,000 DHs per year and provide DH production services upon request at a relatively low cost (25–40 USD in 2018). Private companies have also announced the construction of DH facilities that can have capacity to produce 300,000 DHs per year (https://www.fwi.co.uk/arable/breeders-aim-to-cut-time-to-develop-wheat-varieties), and public-private collaborations such as Heartland Plant Innovations (http://www.heartlandinnovations.com/) have announced the promotion of collaboration in wheat DH (Barkley and Chumley 2012). If there were 200 F_2 plants in a cross, it could result in 1500 crosses in 300,000 DHs.

6.6 How to Apply DH to Wheat Breeding

It has been under debate which breeding scheme is more efficient to apply DH technology into wheat breeding programs and one that can also maximize its benefit to variety development and farmers' benefits. The most fundamental question is which generations will be the best to use for DH production. It is obvious that the application of DH to an F_1 generation can save the time of a breeding cycle, but it will create a huge amount of junk plants without selections in F_2 and F_3 generations. Li et al. (2013) showed the theoretical background to this question, saying that DHs will have less genetic gain than conventional breeding, but DH can have more breeding cycles and have more the gain over a fixed time. Most varieties were developed using F_1 generation (Table 6.1), without any selection prior to DH production. This indicates that the breeders also see the important value of DH for shortening the breeding time. If we look at the number of DHs used in developing the varieties, it ranges from 49 to 863 (Table 6.1). Since the number of the segregating plant in F_2 is 200–500 plants at CIMMYT, the most DH populations are within this range. It is interesting that four varieties were developed from an even smaller population size—less than 100 DH plants: Gk Delibab (49 DH's), UI Sparrow (51 DH's), Florin (64 DH's), and AAC Elevate (84 DHs). This contrasts a few wheat varieties

Table 6.1 The list of doubled haploid-derived wheat varieties

Production method[a]	Variety name	Country	Year	# of DHs[b]	Generations used for DH[c]	Type[d]	References
Anther	Jinghua 1	China	1980s	?		W	Hu et al. (1983)
″	Lung Hua 1	″	″	?		W	
″	Yunhua 1	″	″	?			
″	Yunhua 2	″	″	?			
″	Huapei 764	″	1988	?			Hu et al. (1988)
″	Florin	France	1985	64	F_3	W	De Buyser et al. (1987)
″	Gk Delibab	Hungary	1992	49	F_2	W	Pauk et al. (1995)
″	McKenzie	Canada	1997	?	F_1	S	DePauw et al. (2011a)
″	AC Andrew	″	2001	?		S	″
″	Bam	Iran	2008	?	?	S	Vahabzadeh et al. (2009)
″	Kharoba	Morocco	2010	565	F_1	S	Elhaddoury et al. (2012)
Maize	Faur F	Romania	2004	?	F_1	W	Saulescu et al. (2012)
″	Grandeur	″	2005	?	F_1	D	″
″	Glosa	″	″	?	F_1	W	″
″	Litera	″	2010	?	F_1	W	″
″	Miranda	″	2011	?	F_1	W	″
″	BRS 328	Brazil	2012	?	F_1 or F_2	S	″
″	Kanata	Canada	2000	?	F_1	S	Ref #1[e], Humphreys et al. (2006)
″	Snowbird	″	″	?	F_1	S	Ref #1[e], Humphreys et al. (2007)
″	AC Superb	″	2001	?	F_1	S	Ref #1[e], Townley-Smith et al. (2010)
″	Bhishaj	″	2002	?	F_5	S	Ref #1[e], Randhawa et al. (2011)
″	Lillian	″	2003	?	$BC2F_1$	S	Ref #1[e], DePauw et al. (2005)
″	Burnside	″	2004	?	$BC1F_1$	S	Ref #1[e], Humphreys et al. (2009)
″	Alvena	″	2006	199	F_1	S	Ref #1[e], Knox et al. (2008)
″	CDC Abound	″	2007	?	?	S	Ref #1[e]

(continued)

Table 6.1 (continued)

Production method[a]	Variety name	Country	Year	# of DHs[b]	Generations used for DH[c]	Type[d]	References
″	Waskada	″	″	863	BC_1F_1	S	Ref #1[e], Fox et al. (2009)
″	Stettler	″	2008	168	F_1	S	Ref #1[e], DePauw et al. (2009)
″	Glencross	″	″	?	?	S	Ref #1[e]
″	Accipiter	″	″	?	F_1	W	Ref #1[e], Fowler (2011)
″	Peregrine	″	″	?	F_1	W	Ref #1[e], Fowler (2010)
″	Shaw	″	2009	384	F_1	S	Ref #1[e], Fox et al. (2013)
″	Carberry	″	″	649	F_1	S	Ref #1[e], DePauw et al. (2011b)
″	Muchmore	″	″	649	F_1	S	Ref #1[e], DePauw et al. (2011c)
″	Sunrise	″	″	?	F_1	W	Ref #1[e], Fowler (2012)
″	AC Snowstar	″	″	733	F_1	S	Ref #1[e], Humphreys et al. (2013)
″	Broadview	″	2010	358	F_2	W	Ref #1[e], Graf et al. (2012b)
″	Transcend	″	2011	799	F_1 top	D	Singh et al. (2012)
″	Flourish	″	″	287	F_2	W	Graf et al. (2012a)
″	OAC Emmy	″	″	?	F_1	W	Tamburic-Ilincic and Smid (2013a)
″	AC Emerson	″	2012	171	F_1	W	Graf et al. (2013)
″	OAC Flight	″	″	?	F_1	W	Tamburic-Ilincic and Smid (2013b)
″	Pintail	″	″	?	F_1	W	Salmon et al. (2015)
″	Swainson	″	2013	?	F_1	W	Fowler (2013)
″	CDC Chase	″	″	?	F_1	W	Fowler (2014)
″	UGRC Ring	″	2014	110	F_1	W	Tamburic-Ilincic and Smid (2015)
″	AAC Durafield	″	″	306	F_1	D	Singh et al. (2016)
″	AAC Connery	″	″	543	F_1	S	Canadian Food Inspection Agency[f]
″	AAC Elevate	″	2015	84	F_1	W	Graf et al. (2015)

(continued)

Table 6.1 (continued)

Production method[a]	Variety name	Country	Year	# of DHs[b]	Generations used for DH[c]	Type[d]	References
″	AAC Icefield	″	″	279	F_1	W	Graf et al. (2018)
″	AAC Prevail	″	2017	605	F_1	S	Kumar et al. (2018)
″	Avery	USA	2015	?	F_1	W	Haley et al. (2018a)
″	Langin	″	2016	?	F_1	W	Haley et al. (2018b)
″	UI Sparrow	″	″	51	F_1	W	Chen et al. (2018)
Imperata cylindrica	Him Pratham	India	2015			S	Chaudhary et al. (2015), Patial et al. (2019)

″ = same as the above
[a]Haploid production method
[b]Number of doubled haploid used
[c]The generation used for DH production
[d]Wheat type (D = durum wheat; S = spring bread wheat, W = winter bread wheat)
[e]DePauw et al. (2011a)
[f]Canadian Food Inspection Agency (http://www.inspection.gc.ca/english/plaveg/pbrpov/cropreport/whe/app00009619e.shtml)

released in Canada that used 10,000 F_2 individuals to screen disease resistance plants (DePauw et al. 2013, 2014; Cuthbert et al. 2016, 2017). The difference probably can be from different breeding targets and nature. Genetic theories tell us that the minimum number of DH plants to combine x number of independent genes or chromosomes is Min. = 2^x (except $x = 1$), and as such, it is necessary to have 200 or 500 DH plants to incorporate or eliminate 8 or 9 independently segregating genes. For 50 DHs, in theory it could be less than 4 genes. If two parents are too different, there will be a large number of junk plants for breeding, which is typically seen in elite × wild crosses (Fig. 6.3). The DH method is more appropriate for more targeted and elite × elite crosses. In this sense, genotypic data and genomic selection for parental selections will become more important for the efficient use of DH breeding.

6.7 Future Improvements in Wheat DH Technology

Two recent findings in wild crossing can improve wheat DH production in the future: the gene identification of maize inducer gene and CEN3 mutations. In the maize method, embryo rescue is the most labor intense step, and 50 spikes is the maximum number that one person can handle in a day. Therefore, skipping this process will largely improve DH production in wheat. In this sense, maize is unique

Fig. 6.3 A DH population derived from synthetic wheat (wild type) × OPATA (elite bread wheat). Many junk plants for breeding are present such as the plant in the front left

in doubled haploid production, because it has intraspecific maize inducer lines. When the inducer lines are used as a male parent to cross with another maize line, all chromosomes of inducer lines will be eliminated in a week, leaving the haploid of a female parent (Zhao et al. 2013). This elimination may be due to chromosome fragmentation that could be occurring post meiosis in pollen cells (Li et al. 2017). The original maize inbred line 'stock 6' can induce about 2% haploid formation if it is used as pollen (Coe 1959), but some current inducer maize lines have up to a 16% haploid induction rate (Prigge et al. 2011, 2012), which is close to the embryo setting rate (embryos/pollinated florets) in wheat × maize of many reports (Inagaki and Tahir 1990; Inagaki and Mujeeb-Kazi 1995; Ushiyama et al. 2007; Hussain et al. 2012). Recently, a candidate gene for the haploid induction was identified as MATRILINEAL which encodes a sperm-specific phospholipase (Gilles et al. 2017a; Kelliher et al. 2017). An even more important finding is that mutation in MATRILINEAL homolog in rice also induced haploid formation for an average of 6% (Yao et al. 2018). In sorghum, two natural inducer lines were found by screening 4000 germplasms worldwide (Hussain and Franks 2019). Since MTL is highly conserved in cereals (Kelliher et al. 2017), it may be possible to find mutations in each wheat genome and combine them to construct a "wheat haploid inducer line."

A centromere-specific histone CENH3 is another possibility. Many researchers have thought that chromosome elimination might be caused by centromeric deficiency between wheat and maize. This idea was recently supported by a centromere-mediated genome elimination in *Arabidopsis thaliana* (Ravi et al. 2001). In the study, they mutated a centromere-specific histone CENH3 and observed that the

chromosomes entering the zygote from the mutant were eliminated during the hybrid embryo development (Ravi et al. 2001). A point mutation on barley CENH3 showed a reduced CENH3 loading on centromere (Karimi-Ashtiyani et al. 2017), which can lead to haploid formation. This type of mutation has not yet been identified or developed in wheat, but it is possible in the near future. Viable haploid plant induction rate is a very high at 25–45% (Ravi et al. 2001). Finding mutant or modification of CENH3 can also greatly improve wheat DH production.

6.8 Conclusion

DH technology has already been widely utilized in wheat breeding. New DH-derived wheat varieties have been emerging every year in Western countries, and they cover a significant percentage of wheat fields in Canada (30% in 2011) and Romania (16% in 2012). New DH facilities, which produce more than 50,000 DHs every year from one place, have also been recently opening. These facilities can provide services for wheat breeders who do not have access to DH breeding. Future improvements in DH production may make both the service and technology more accessible and at a lower cost. A key step for world stability and poverty reduction is sustainably producing wheat and supplying to an ever-increasing world population that demands it. This task has been becoming much more difficult due to decreasing annual yields, climate change, and emergence of new diseases. DH technology can bring more genetic gain to achieve greater yield increase and, at the same time, adapt for climate change. It also makes it possible to react emergency problems quickly. It is a key tool for future food security.

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Chapter 7
Recent Advances in Chromosome Elimination-Mediated Doubled Haploidy Breeding: Focus on Speed Breeding in Bread and Durum Wheats

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7.1 Introduction

Ensuring food security for the burgeoning population is the aim of scientific community across the world today. Durum wheat (*Triticum turgidum* L. subsp. *durum*, $2n = 4x = 28$ = AABB genomes) and common wheat (*Triticum aestivum* L., $2n = 6x = 42$ = AABBDD genomes) are two important cereal crops which contribute substantially to global food and nutritional security. Hence, production of wheat has to be boosted to feed the population steadily. But the path of future wheat production poses various challenges including biotic as well as abiotic stress, changes in urbanization pattern and demand for better quality wheat. Genetic variability provides adaptability, that is, the capacity for genetic change in response to selection. Increasing the genetic variability of crop is a prime goal to generate new and superior recombinants. The preferred approach to achieve the goal is through distant hybridization involving parents from secondary and tertiary gene pools of wheat family, which can contribute novel chromatin into wheat background. But the major bottleneck in the successful implementation of the approach is preferential uniparental chromosome elimination during post-zygotic division, leaving the haploid embryos. Such embryos have to be nurtured using tissue culture techniques so as to develop it into a plant. Using artificial chromosome doubling techniques, doubled haploid (DH) plants are produced from haploid plantlets leading to the generation of genetically homozygous plants (Fig. 7.1).

Doubled haploids (DH) produced from uniparental chromosome elimination are indispensable in advanced breeding programmes to accelerate genetic upgradation of wheat through production of stable intergeneric hybrid plants. Newly synthesized allopolyploids (amphiploids) encounter a variety of rapid and reproducible genomic

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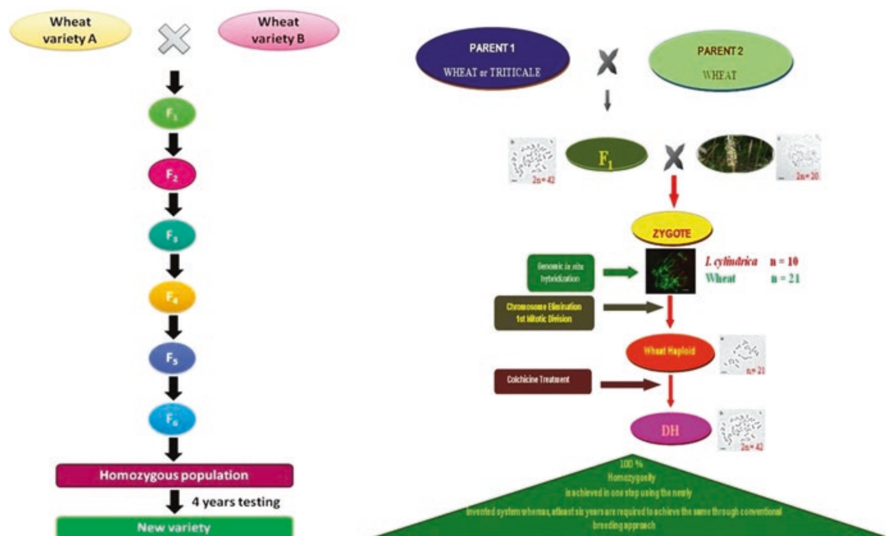

Fig. 7.1 Flow chart exhibiting comparison of the conventional (left flank) and DH breeding (right flank) approaches (Chaudhary et al. 2013b)

changes, including the elimination of DNA sequences (Liu et al. 1998; Ozkan et al. 2001; Shaked et al. 2001; Ma et al. 2004; Pires et al. 2004; Adams and Wendel 2005; Skalicka et al. 2005; Lukens et al. 2006; Gaeta et al. 2007). Haploid technology has tremendous use for accelerating breeding technologies when combined with marker-assisted selection (MAS). It is possible to pyramid resistance genes by combining MAS and DH breeding technology. Due to complete homozygosity in doubled haploids, the efficiency of selection for both qualitative and quantitative characters is increased. DHs with less frequent alleles can be produced and developed for breeding purposes. The task becomes easier when reliable molecular markers for the desired traits have been identified. Selection can be performed at an early stage to eliminate undesirable recombinants among progenies of suitable crosses, thus saving time, labour and space. In the present chapter, the authors will try to uncover the mechanisms of chromosome elimination behind DH technology and how the breeders have utilized it in the upgradation of genetic makeup of wheat worldwide. Simultaneously, the future prospects of the technique will be discussed.

7.2 Chromosome Elimination: Mechanism

Distant hybridization is one of possible in vivo approaches to transfer desirable traits from wild species into cultivated species. However, these hybridization endeavours are affected by various pre-fertilization and post-fertilization barriers.

Pre-fertilization barriers include failure of zygote formation due to pollen-stigma incompatibility and failure of pollen tube to reach the ovary, whereas post-fertilization barriers comprise failure of zygote development and uniparental or preferential chromosome elimination. The phenomenon of chromosome elimination has been observed in several interspecific hybrids (Kasha and Kao 1970; Barclay 1975; Laurie and Bennett 1988a, b, 1989; Riera-Lizarazu et al. 1996; Fujiwara et al. 1997; Chaudhary et al. 2005). Earlier it was thought to be an obstacle in transferring the genes from alien species into crop plants. But in the past few decades, this obstacle has turned out into boon in breeding programmes for induction of haploids in crop plants (Devaux and Pickering 2005). Many researchers across the world proposed different reasons for this phenomenon. After interspecific fertilization, two different parental genomes are enclosed and mixed within one nucleus, which is embedded in the maternal cytoplasm, mostly. Such genomic constitution may result in intergenomic conflicts leading to genetic and epigenetic reorganization (Riddle and Birchler 2003). Even if in most cases the parental genomes remain combined after a successful fertilization, an elimination of specific DNA sequences frequently follows in the early stages of allopolyploidization (Feldman et al. 1997; Liu et al. 1997). In grasses, a partial somatic elimination of chromosomes from one parental species may occur, for example, in wide crosses of *Hordeum lechleri* × *H. vulgare* (Linde-Laursen and von Bothmer 1999), *Avena sativa* × *Zea mays* (Riera-Lizarazu et al. 1996) or *Triticum aestivum* × *H. vulgare* (Barclay 1975). Complete uniparental chromosome elimination also occurs in some interspecific hybrids between closely related species (as *H. vulgare* or *H. parodii* × *H. bulbosum* and *H. marinum* × *H. vulgare*) (Kasha and Kao 1970; Subrahmanyam 1977; Finch 1983) as well as between remotely related parental species (*Aegilops* spp., ryegrass (*Lolium multiflorum*), barley, oat, rye (*Secale cereale*), or wheat × *Pennisetum glaucum*, *Sorghum bicolor*, *Tripsacum dactyloides*, or *Z. mays*) (Zenkteler and Nitzsche 1984; Laurie and Bennett 1986, 1988a; Rines and Dahleen 1990; Chen et al. 1991; Matzk and Mahn 1994; Matzk 1996; Matzk et al. 1997). The uniparental chromosome elimination may be due to the asynchronous cell cycles that led to disparity in timing of essential mitotic processes (Gupta 1969) and related to mitotic rhythm and the duration of cell cycle phases (Lange 1971). The chromosome elimination is also associated with formation of multipolar spindles (Subrahmanyam and Kasha 1973) and asynchrony in synthesis of nucleoprotein that leads to loss of the most retarded chromosomes (Laurie and Bennett 1989). Additionally, the role of species- and genotype-specific nucleases in degradation of alien chromosomes (Davies 1974), spatial separation of genomes during interphase (Linde-Laursen and von Bothmer 1999), deficiency of parent-specific factors on kinetochores responsible for chromosomes movement (Mochida et al. 2004) and elimination of genome by formation of nuclear extrusions during interphase in addition to post-mitotically formed micronuclei (Gernand et al. 2006) are the other proposed factors for uniparental chromosome elimination in interspecific hybridization. Some other theories involved in this phenomenon are due to the lack of kinetochore activity, which leads to missegregation of chromosomes in mitosis

(Komeda et al. 2007), the role of centromere-specific histone mutants (Ravi and Chan 2010) and uniparental non-disjunction of anaphase chromosomes (Ishii et al. 2010). CENH3, a variant of the centromere-specific histone H3, is also considered a major key point in uniparental chromosome elimination during wide hybridization programme (Ishii et al. 2016).

Approaches for haploid generation through chromosome elimination have been described as follows.

7.2.1 Wide Hybridization

7.2.1.1 Bulbosum Method

Hordeum vulgare × *H. bulbosum* wide hybridization method, commonly known as bulbosum method, was at first reported in cereals based on chromosome elimination (Stephan 1969; Kasha and Kao 1970; Lange 1971). The chromosomes of the wild relative, *H. bulbosum* ($2n = 2x = 14$), were reported to be preferentially eliminated from the cells of developing embryos during early embryogenesis leading to the formation of haploid embryos. Kasha and Kao (1970) presented evidence to show that these haploids are produced by the elimination of *H. bulbosum* chromosomes and not by parthenogenesis. The endosperm is frequently formed, but its development is usually interrupted leading to embryo abortion. Hence, embryo rescue technique or in vitro culture plays a vital role development of embryos produced from distant hybridization. This bulbosum approach was used in breeding programmes to produce large number of haploids in most genotypes. Comparative efficiency of bulbosum technique for haploid induction in barley was as high as compared to anther culture.

The potential of this method was exploited in wheat where the androgenesis-mediated haploid induction response was very poor due to genotype specificity (Barclay 1975). When ‘Chinese Spring’ variety of *T. aestivum* ($2n = 6x = 42$) was crossed with *H. bulbosum* ($2n = 2x = 14$), haploid wheat plantlets were obtained as a result of elimination of *H. bulbosum* chromosomes from the interspecific hybrid during its early embryogenesis (Barclay 1975; Zenketler and Straub 1979). But in other wheat varieties, crossability with *H. bulbosum* was restricted by dominant crossability inhibitor alleles *Kr1*, *Kr2*, *Kr3* and *Kr4* located on 5B, 5A, 5D and 1A (Riley and Chapman 1967; Krolow 1970; Sitch et al. 1985; Zheng et al. 1992). These alleles prevent the entry of *H. bulbosum* pollen tube into the ovary of wheat. The presence of their recessive alleles in ‘Chinese Spring’ made this variety successfully crossable with *H. bulbosum*. Some loci found on chromosomes 3A, 3B and 3D were also found responsible for crossability between ‘Chinese Spring’ wheat and *H. bulbosum* (Miller et al. 1983). The sensitivity of the *H. bulbosum* pollen to the crossability inhibitor genes limited its application in wheat breeding programmes.

7.2.1.2 Wheat × Maize System

Zenkteler and Nitzsche (1984) were pioneers to report haploid embryo formation from the crosses between hexaploid wheat and diploid maize. Laurie and Bennett (1986) confirmed their results cytologically by demonstrating that maize pollen normally germinated and grew into wheat embryo sac where wheat egg was fertilized by the maize pollen. A hybrid zygote having 21 wheat chromosomes and 10 maize chromosomes was produced (Laurie and Bennett 1988a, b). Hybrid zygotes were karyotipically unstable, and during cell divisions, the maize chromosomes failed to move towards the spindle poles due to the progressive loss of centromere activity of maize chromosomes and lack of their attachment to the spindle microtubules. As a result of this, maize chromosomes were eliminated after three to four mitotic cell divisions forming wheat haploid embryo with $n = 21$ chromosomes (Laurie and Bennett 1989). The maize was reported to be insensitive to the action of dominant genes *Kr1* and *Kr2*, located on the long arms of chromosome 5B and 5A, respectively (Sitch et al. 1985). Due to the insensitivity of maize pollen to the crossability inhibitor genes, this method could be applied to a wide range of wheat as well as maize genotypes including those recalcitrant to androgenesis (Cherkaoui et al. 2000; Chaudhary et al. 2002; Singh et al. 2004; Pratap et al. 2006). Haploid production efficiency is influenced by the proportion of pollinated florets which develop haploid embryos. Production of haploid embryos has been reported to be as high as 53% (Morshedi and Darvey 1995) and as low as 1% (Suenaga and Nakajima 1989) depending upon a wide range of variables.

Pratap et al. (2006) evaluated comparative efficiency of anther culture and wheat × maize system for embryo formation and haploid plantlet regeneration in wheat and triticale genotypes and found maize-mediated system of haploid induction significantly better than androgenesis. The advantage of wheat × maize hybridization technique over anther culture and the bulbosum technique in terms of reduced genotypic specificity, absence of albinism and ease of application makes it more efficient for the production of haploids in common wheat (Wang et al. 1991). The wheat × maize system was efficiently utilized in the development of the first doubled haploid wheat variety of India—Him Pratham (Chaudhary et al. 2013b) (Fig. 7.3)—by Dr. Harinder Kumar Chaudhary and his associates, CSK HP Agricultural University, Palampur, Himachal Pradesh, India (Fig. 7.2).

Durum wheat ($2n = 4x = 28$) or macaroni wheat is the tetraploid species of wheat of commercial importance that is widely cultivated today. The ploidy level is not a barrier in the production of haploid embryos through wheat × maize system, and haploids have been produced in durum wheat using maize as the pollen source (Ahmad and Chowdhry 2005). Haploid seedlings were recovered from *Triticum turgidum* ssp. *turgidum* cv 'Rampton Rivet' pollinated with maize following in vivo treatment of ovaries with 2,4-D for 2 weeks and subsequent embryo culture. The high haploid induction efficiency and genotype non-specificity of wheat × maize system in comparison to anther culture and bulbosum technique make the system

Fig. 7.2 First doubled haploid wheat variety of India: DH 114 (Him Pratham) developed through chromosome elimination-mediated approach (Chaudhary et al. 2013b)

Fig. 7.3 Spike of a wildly growing plant of *Imperata cylindrica*, an efficient pollen source for haploid induction in wheat

more practicable. However, the flowering times of maize and wheat coincide under field conditions in subtropical and tropical climates, while in other areas experiments are run under glasshouse conditions.

Some of the alternative pollen sources for haploid induction in wheat were explored, but no alternative pollen source was reported to overcome the problems of wheat × maize system, viz. non-synchronization of flowering with wheat naturally and poor performance in producing haploids from triticale × wheat and wheat × rye derivatives (Kishore et al. 2011). These constraints made it imperative to search for

some other pollen source. Among all the Gramineae genera, viz. *Zea mays*, *Sorghum bicolor*, *Pennisetum americanum*, *Setaria italica*, *Festuca arundinacea*, *Imperata cylindrica*, *Cynodon dactylon*, *Lolium temulentum* and *Phalaris minor* tested for haploid plant production, *Imperata cylindrica* has emerged as more efficient and significantly superior over wheat × maize system for doubled haploid production in wheat (Chaudhary et al. 2005; Pratap et al. 2005).

7.2.1.3 Wheat × *Imperata cylindrica* System

Wheat × maize system was reported to have serious constraints like non-synchronization of flowering with wheat naturally and poor performance in producing haploids from triticale × wheat and wheat × rye derivatives. Considering the above chromosome elimination-mediated haploid induction systems, no alternative pollen source was reported to overcome the problems of wheat × maize system. *Imperata cylindrica*, a wild weedy perennial grass ($2n = 2x = 20$), coincides well for flowering with that of wheat and triticale under natural conditions and is available in almost all parts of the world wherever wheat is cultivated (Fig. 7.3).

The *I. cylindrica* has emerged as an efficient alternative pollen source for doubled haploid production in wheat that has been found to be significantly superior over maize-mediated haploid induction technique (Chaudhary et al. 2005). Intergeneric crosses between *I. cylindrica* and wheat followed by elimination of *I. cylindrica* genome has emerged as the system of choice for inducing haploids not only in bread wheat (Chaudhary et al. 2005, 2013a, b; Chaudhary 2008a, b, 2009, 2010a, b, 2012, 2013a, b; Tayeng et al. 2012) but also in durum wheat (Mahato and Chaudhary 2015). This potential pollen source is genotype non-specific and is able to induce haploids in any genotype of wheat and triticale or their derivatives. Wheat × *I. cylindrica* is more efficient, effective, fast, having synchronization in flowering and above all the simple technique for haploid induction when compared with wheat × maize-mediated haploid development technique (Chaudhary 2010a). *I. cylindrica* has been reported to perform significantly better than maize for all the haploid induction parameters in wheat and triticale and their derivatives (Fig. 7.4) (Chaudhary 2008a, b, 2012, 2013a, b; Kishore et al. 2011; Jeberson et al. 2012; Chaudhary et al. 2013a, b; Badiyal et al. 2014; Rather et al. 2014, 2017).

Cytological investigation of the wheat × *I. cylindrica* chromosome elimination system has shown that there is no endosperm formation, and the elimination of chromosomes of *I. cylindrica* takes place in the first zygotic division during the process of seed development, thus allowing the production of embryo-carrying pseudoseeds (Komeda et al. 2007) (Fig. 7.5) . Kaila et al. (2012) reported that the chromosome elimination in wheat × *I. cylindrica* system is being triggered by the B and D genome of wheat. Tayeng et al. (2012) reported the enhancement in doubled haploid production efficiency in wheat × *I. cylindrica*-mediated chromosome elimination approach of doubled haploidy breeding following in vivo (0.02%) application of colchicine. Sharma et al. (2019) standardized the protocol for in vitro

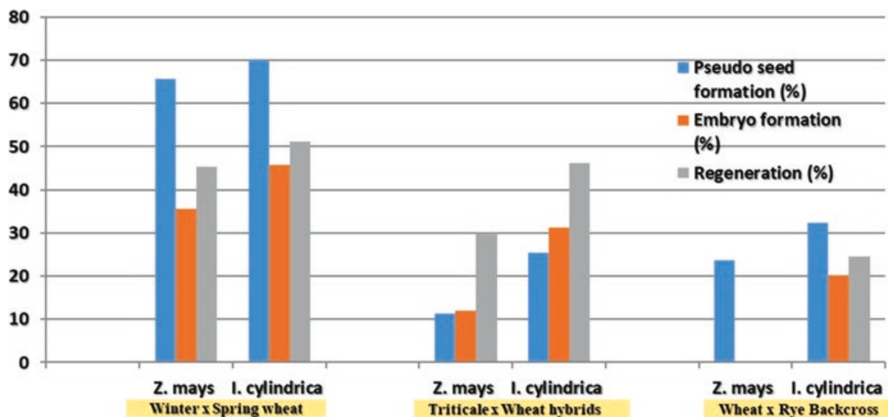

Fig. 7.4 Comparative efficiency of maize and *I. cylindrica* for haploid induction parameters in various inter- and intraspecific wheat hybrids (Chaudhary 2008a, b; Kishore et al. 2011)

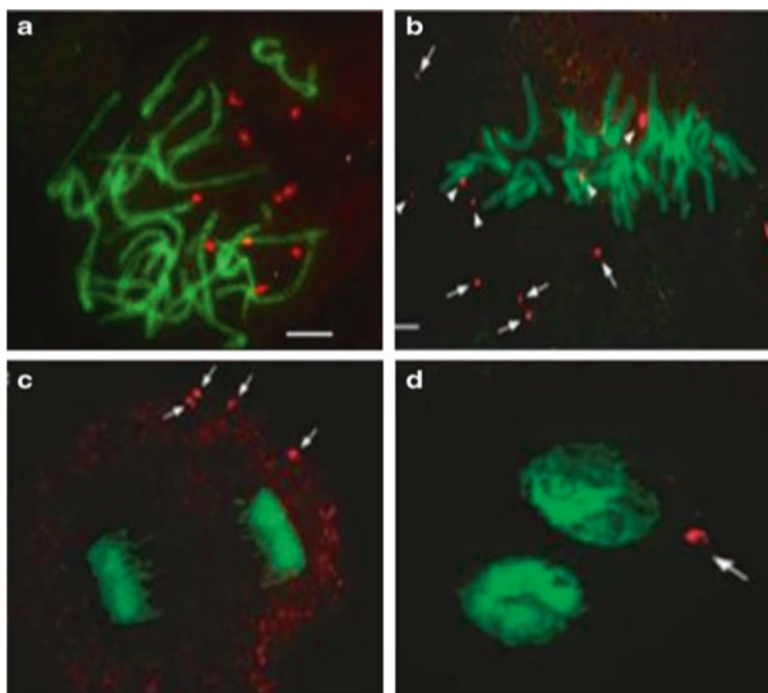

Fig. 7.5 Molecular cytological evidence of sequential elimination of alien chromosomes in wheat × *I. cylindrica* hybrids during first zygotic mitosis (green, wheat chromosomes; red, *I. cylindrica* chromosomes). (**a**) Interspecific hybrid, (**b**) aberrant movement of *Imperata* chromosomes, (**c**) anaphase cell showing eliminating of *Imperata* chromosomes, (**d**) interphase cells after 3–4 days of pollination showing extruded *Imperata* micronucleus (Komeda et al. 2007)

application of colchicine to enhance doubled haploid production in hexaploid (0.075% for 4 h) and tetraploid wheat (0.15% for 4 h).

The efficiency of wide hybridization for haploid induction depends primarily on the functional viability and relative longevity of pollens of the parent. It has been reported that maize pollen loses its viability completely within 50 min (Luna et al. 2001; Aylor 2004; Muui et al. 2007). An investigation was conducted for comparative assessment of the viability and longevity of freshly harvested pollen of maize and *I. cylindrica*. *I. cylindrica* pollen, being viable for significantly longer period than maize, can accelerate the haploid induction endeavours (Mayel et al. 2015). The relative efficiency for induction of haploids in durum wheat through maize- and *I. cylindrica*-mediated chromosome elimination was studied by Mahato and Chaudhary (2015) using two genotypes of maize and *I. cylindrica* as pollen sources and seven durum wheat genotypes utilized as maternal parent. The response of *I. cylindrica* was significantly higher as compared to maize in all the haploid induction parameters, and hence it appeared to be the most efficient source for induction of haploids in *T. durum* (Fig. 7.6).

Anthesis in wheat spikes initiates from the upper middle portion of the spike and progresses bi directionally. This asynchronous behaviour of anthesis within wheat spikes can be efficiently utilized for haploid induction by pollinating the spikes with *I. cylindrica* pollen without emasculation (Chaudhary et al. 2013a, b). Morphological marker, that is, the absence of endosperm in wide hybrid seeds, can be utilized for differentiating normal and haploid embryo-carrying seeds using incandescent bulbs (Fig. 7.7).

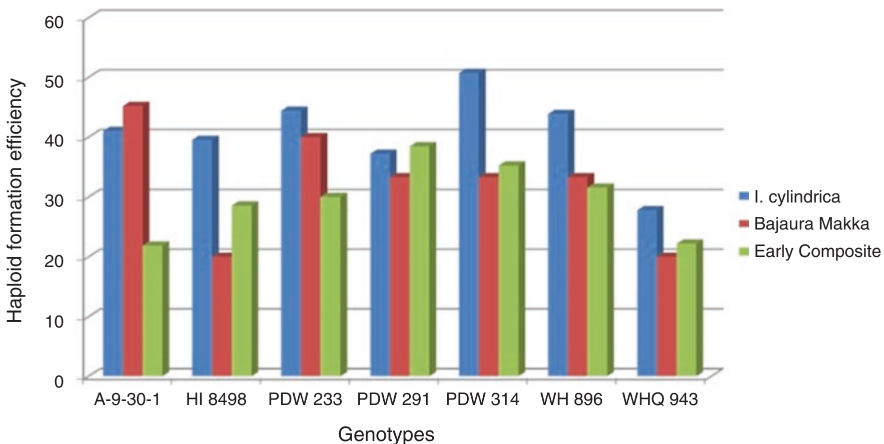

Fig. 7.6 Relative efficiency of different pollen sources on haploid formation efficiency in durum wheat (Mahato and Chaudhary 2015)

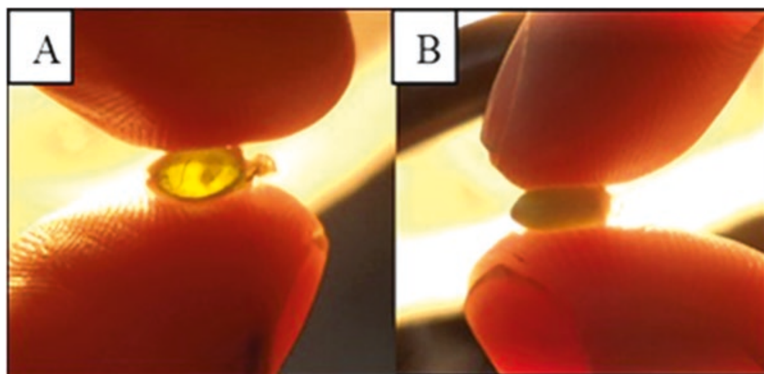

Fig. 7.7 (**a**) Hybrid seed containing embryo devoid of endosperm. (**b**) Selfed seed-containing endosperm (Chaudhary et al. 2013b)

7.2.2 Targeted Centromere Manipulation

The haploid induction technologies have one common limitation that particular technology is limited to particular crop genotypes and species. The stepping stone to overcome this limitation was laid by Finch (1983) who revealed that in the chromosome elimination technique, the centromeric constrictions of eliminated *Hordeum* chromosomes were always either absent or much smaller than those of the retained chromosomes. This finding provided the first association between centromere function and uniparental chromosome elimination. This discovery laid a foundation in the possibility of producing haploids through centromere modifications. Finally, Ravi and Chan (2010) described a novel method of in vivo haploid induction which can be exploited across species, through CENH3-modified centromere-mediated genome elimination.

The centromeres are the assembly sites for the kinetochore, a multi-protein complex to which spindle microtubules are attached at mitosis and meiosis, thereby ensuring segregation of chromosomes during cell division (Jiang et al. 2003). Plant and animal centromeres are epigenetically specified by a centromere-specific variant of histone H3, CENH3 (or CENP-A), which replaces canonical histone H3 in the nucleosomes of functional centromeres (Houben and Schubert 2003). Modifications in CENH3 gene transcription or translation could affect the ability to assemble intact CENH3 chromatin and might result in the loss of CENH3 from the centromere region and a loss of proper centromere function (Wang et al. 2019). Contrary to canonical histone H3, which is extremely conserved, CENH3 shows considerable variability between species and shows some signs of adaptive evolution (Malik and Henikoff 2009).

CENH3 consists of an N-terminal tail region, which is highly variable even between closely related species and a C-terminal histone fold domain (HFD), which is well conserved across species (Malik and Henikoff 2003; Kuppu et al. 2015;

Britt and Kuppu 2016). The N-terminal tail has one alpha helix (αN), and the HFD has three alpha helices separated by two loop regions (α1-L1-α2-L2-α3) (Ishii et al. 2015; Watts et al. 2016). The CENP-A targeting domain (CATD), composed of loop 1 and α2, is necessary for CENH3 loading to the centromere (Sullivan et al. 1994; Lermontova et al. 2006; Black et al. 2007).

Ravi et al. (2010) found that transgenic green fluorescent protein-tagged (GFP) CENH3 (GFP-CENH3) was able to complement the phenotype of CENH3-1, an embryo-lethal null mutant, although some lines showed reduced complementation. The results indicate that the GFP tag affects the function of centromeres. Further, to construct a chimeric H3.3/CENH3 protein, the N-terminal tail of CENH3 was replaced by the tail of a conventional *Arabidopsis* histone H3. Then, the protein fused with a GFP (green fluorescent protein) reporter to construct the GFP-tailswap protein, which could rescue CENH3-1 mutants (Ravi and Chan 2010). However, GFP-tailswap plants were mostly male sterile, attributable to meiosis defects. After self-pollination, GFP-tailswap plants produced normal diploid seed at about 1% of the normal rate (Ravi and Chan 2010; Ravi et al. 2011). On outcrossing, the chromosomes from GFP-tailswap were frequently lost post-fertilization producing haploids and aneuploids. When crossed with wild-type plants as female parent, GFP-tailswap plants produced 25–45% haploids and 28–50% aneuploids. These rates were reduced to 4–5% for haploids and 4–11% for aneuploids when the mutant line is used as male parent. The efficiency of modified or altered CENH3 increased many fold when it is used as female in the haploid induction as compared to when used as male (Ravi and Chan 2010; Karimi-Ashtiyani et al. 2015)

The initial findings with GFP-CENH3 and GFP-tailswap suggested that addition of a bulky tag to CENH3 might interfere with CENH3 recognition and so produce a centromere that is less competitive for the reloading of CENH3 or subsequent components of the centromere when confronted with competing wild-type centromeres. Based on this hypothesis, these modified CENH3s and their resulting centromeres were designated as "weak" or "non-competitive" vs. wild-type CENH3. Addition of GFP on its own presumably results in a slightly defective protein, and modification of the N-terminal tail (tailswap) further degrades the strength of the resulting centromere. The addition of adducts such as GFP and changes in the sequence of CENH3 can act additively to affect protein function. For example, as described above, GFP-CENH3, in *Arabidopsis*, is a haploid inducer, but otherwise fully functional. The *Arabidopsis* null mutant can also be complemented (mitotically and meiotically) by CENH3 from a wide range of angiosperms (Maheshwari et al. 2015) including the monocot *Zea mays* (Ravi et al. 2010).

7.2.2.1 Methods of CENH3 Modifications

The alterations in CENH3 can be done in three basic ways, first by modifying its N-terminal tail, second by modifying highly conserved histone fold domain (HFD) and finally by replacing whole CENH3 with its ortholog from related species. The tailswap of *A. thaliana* N-terminal tail between less related species causes severe

sterility due to defective centromeres (Ravi et al. 2014), implying that altering the N-terminal tail composition has the potential to create haploid inducer. In addition to the simple conjugation of the GFP tag at the N-terminus (GFP fusion), replacement of the N-terminal tail with H3.3 N-terminal tails with or without a GFP tag has been shown to induce haploid formation (Ravi and Chan 2010; Ravi et al. 2011; Kelliher et al. 2017). Attempts by N-terminal tail editing have been performed in diverse species (Britt and Kuppu 2016), and haploid induction has been successfully reported in maize (0.065–0.86%) (Kelliher et al. 2017) and rice (0.3–1.0%) (Kalinowska et al. 2019), which demonstrated the feasibility of haploid induction by engineering the CENH3 N-terminal tail in monocotyledonous crop plants.

In wheat, previously only one *CENH3* gene was encoded (Li et al. 2013). But Yuan et al. (2015) revealed two types of *CENH3* genes, named α*CENH3* and β*CENH3*, and each has three slightly different copies derived from the AA, BB and DD genomes. In most tetraploid wheat species, *CENH3* genes are more highly expressed from the AA genome. In wild tetraploids, β*CENH3* has a much lower expression level than α*CENH3*, while in cultivated tetraploids, β*CENH3* transcripts are enhanced to near α*CENH3* levels. Comparison of the CENH3 proteins in wild and cultivated tetraploids revealed that the histone fold domain (HFD) of only β*CENH3* is under positive selection, especially in the region responsible for targeting of CENH3 to the centromere. There is still no report of modification in *CENH3* gene of wheat.

Yang et al. (2019) synthesized a hybrid wmCENH3 gene, which has the N-terminus before loop 1 domain from wheat TaCENH3, the C-terminal HFD after loop 1 from maize ZmCENH3 and a red fluorescent protein (RFP) tag at the C-terminus. The synthesized wmCENH3 gene was cloned into a gene expression cassette under a strong maize ubiquitin promoter (Christensen and Quail 1996) and transformed into wheat by biolistic transformation. Transgenic wheat was generated, and the chromosome behaviours in wheat/maize somatic hybrids revealed mislocalization of both ZmCENH3-YFP and wmCENH3-RFP which further may cause centromere inactivation.

7.2.3 CRISPR/Cas9-Mediated Targeted Chromosome Elimination

The alteration in any genomic sequence or gene is possible by inducing unique double-strand breaks (DSBs) by a site-specific endonuclease at the sites of interest. For this purpose, synthetic nucleases, namely, zinc finger nucleases (ZFNs), in 2003 and transcription activator like effector nucleases (TALENs) in 2010 have been developed for the targeting of genomic sites (Doudna and Charpentier 2014). Although zinc finger nucleases (ZNF) solved the basic problem of targeting DSBs to specific site in the genome, still the system revealed numerous drawbacks like time-consuming construction of the enzymes and high cost, and their limited specificity resulted in unwanted secondary ‘off-target’ mutations. These limitations were

overcome by TALENs (transcription activator-like effector nucleases) which have higher specificity as well as low cost and time requirements. But still there was less adaptation for TALENs due to the requirement of different dimeric proteins specific for each target site, which limits their practical use for multiplexing (Chen et al. 2019).

Although CRISPR arrays were first identified in the *Escherichia coli* genome in 1987 (Ishino et al. 1987), their biological function was not understood until 2005, when it was shown that the spacers were homologous to viral and plasmid sequences suggesting a role in adaptive immunity (Bolotin et al. 2005; Mojica et al. 2005; Pourcel et al. 2005). In 2012, the transition of the CRISPR/Cas system from biological phenomenon to genome engineering tool took place when it was shown that the target DNA sequence could be reprogrammed simply by changing 20 nucleotides in the crRNA and that the targeting specificity of the crRNA could be combined with the structural properties of the tracer RNA in a chimeric single guide RNA (gRNA), thus reducing the system from three to two components (Jinek et al. 2012). In 2013, the first application of CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat)-based genome editing in plants has been reported by three independent groups in rice (*Oryza sativa*), *Nicotiana benthamiana* and *Arabidopsis thaliana* (Li et al. 2013; Nekrasov et al. 2013; Shan et al. 2013) to generate small deletions, targeted insertions and multiplex genome modifications. This provides plant breeders a widespread ability to control the specific introduction of targeted sequence variation, which provides a game-changing resource for rapid improvement of agricultural crops. The CRISPR/Cas system has made genome editing a widely adopted, low-cost, easy-to-use targeted genetic manipulation tool that has been applied to many crops. Bhowmik et al. (2018) combined the CRISPR/Cas9 system with microspore technology and developed an optimized haploid mutagenesis system to induce genetic modifications in the wheat genome and found that electroporation of a minimum of 75,000 cells using 10–20 μg DNA and a pulsing voltage of 500 V is optimal for microspore transfection using the Neon transfection system. Using multiple Cas9 and sgRNA constructs, they presented evidence for the seamless introduction of targeted modifications in an exogenous *DsRed* gene and two endogenous wheat genes, including *TaLox2* and *TaUbiL1*. Liu et al. (2019) developed double knockout mutants in wheat for *MTL/ZmPLA1/NLD* homologue in *qhir1*, a gene known for triggering haploid induction in maize (Gilles et al. 2017; Kelliher et al. 2017; Liu et al. 2017), and observed a hike in wheat haploid induction frequency by 2–3%, accompanied by a rise of 30–60% in seed setting rate.

7.3 Chromosome Doubling for Generation of Homozygous Plants from Haploids

Haploids in some crop species such as barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.), show spontaneous chromosome doubling as high as 65–76% in barley and 27–83% in rye, irrespective of the genetic background (Immonen and Tenhola-

Roininen 2003; Kahrizi and Mohammadi 2009), which is satisfactory for production of DH lines. But the phenomenon is not observed in other species. Therefore, artificial chromosome doubling using colchicine application has become an integral part of DH technology (Navarro-Alvarez et al. 1994; Prigge and Melchinger 2012; Würschum et al. 2012). Colchicine treatment has become the benchmark for artificial chromosome doubling in cereals because of the high success rate so far. Colchicine prevents formation of microtubules during cell division so that chromosomes are not pulled apart during mitosis, which results in a duplication of the number of chromosomes in the cell (Bartels and Hilton 1973; Wan et al. 1991; Chaikam et al. 2012). However, colchicine is highly toxic (Terkeltaub 2009) and potentially carcinogenic. Hence, in addition to proper application, utmost care must be taken by the users of colchicine in storage, handling and personal protection measures. Moreover, colchicine is hazardous to the environment and requires proper disposal after treatment. Such safety measures are expensive and difficult to implement in developing countries. Hence various chromosome doubling agents like various herbicides and caffeine are being explored as alternatives (Lehnen Jr and Vaughn 1991). Several herbicides such as APM, pronamide, trifluralin, oryzalin and chlorpropham have been tested for their suitability as doubling agents in maize callus culture (Wan et al. 1991; Beaumont and Widholm 1993) and in maize root tips (Häntzschel and Weber 2010).

7.4 Chromosome Elimination-Assisted Wheat Improvement

Chromosome elimination, whether it is preferential elimination of chromosome segment, whole chromosome/s or whole genome, is one of the preferred techniques which are practised for the development of doubled haploids as well as transgenics. DH breeding is one of the most efficient methods for recurrent selection and line development, especially for low heritable traits (Gallais 1990). DH technology hastens the speed of the cultivar development in inbred crops by instant fixation of inherited traits and makes phenotyping and genotyping more easy and predictive. In doubled haploids, recessive alleles become homozygous in one generation and can be expressed directly. Doubled haploidy breeding using chromosome elimination has been employed in wheat improvement programmes, and new wheat cultivars have been released in many countries such as China, France, Hungary and Canada (Hu et al. 1983; DeBuyser et al. 1987; DePauw et al. 2011). Classical examples of cultivar development using this approach include the development of promising wheat cultivars having agronomic performance comparable to local standard check variety and very good milling characters (Ushiyama 2008). A soft red winter wheat variety 'Kinuhime' was released in 1998, developed by wheat × *H. bulbosum* hybridization method. This variety has traits like early maturity, high yield and sprouting and lodging resistance. Two more wheat cultivars 'Yumeasahi' and 'Hanamanten' were developed by utilizing wheat × maize system, which were released in 2003 and 2004, respectively. Both these varieties are early maturing,

have large ear numbers and have high sprouting resistance. The wheat × maize system was efficiently utilized in the development of the first doubled haploid wheat variety of India—Him Pratham (Chaudhary et al. 2013b)—by Dr. Harinder Kumar Chaudhary, CSK HP Agricultural University, Palampur, Himachal Pradesh, India. Him Pratham is a semidwarf, spreading-type facultative winter wheat showing appreciable resistance to all the rusts and powdery mildew with average grain yield of 37–40q/ha and is recommended for dry and wet temperate regions of the mountainous tracts of northwest Himalayas.

DHs developed by whole genome elimination can be used in a recurrent selection scheme in which superior DHs of one cycle represent parents for hybridization for the next cycle. Gradual improvement of lines is expected due to the alternation of recombination and selection by performing several cycles of crossing, DH production and selection. DH populations can be used as permanent mapping populations because they are stable and constant. DH populations have also been used in the creation of molecular marker maps and quantitative trait locus (QTL) identifications (Chauhan and Khurana 2011; Wu et al. 2012) in wheat. Commercially promising DHs or lines can be genotyped with a set of molecular markers dispersed on each chromosome along with their parents and the most commonly grown cultivars. The genotyping data along with phenotype records help in the choice of potential crosses by strengthening the characterization of new lines as well as determining their relative genetic distance from the current cultivars. Understanding the organization in complex genomes using genetic markers and further manipulation of loci controlling traits of interest represents a major breakthrough for plant geneticists and breeders. The use of DHs in QTL mapping has been demonstrated using simulation studies, particularly when the effect of the QTL is low (Martinez et al. 2002). Wheat DH populations have been used to develop whole genome linkage maps to locate QTLs controlling important agronomic traits (Chu et al. 2008a, 2009; Zhang et al. 2012), number of tillers (Li et al. 2010), number of spikes per plant and spike length (Suenaga et al. 2005), photoperiod response and heading time (Sourdille et al. 2000), crossability among different wheat genomes (Tixier et al. 1998), resistance to foliar disease of wheat (Chu et al. 2008b), *Fusarium* head blight (Yang et al. 2005), leaf rust resistance (Huang et al. 2003; Prins et al. 2011), tolerance to abiotic stresses (Hill et al. 2013; Gahlaut et al. 2017) and quality (Perretant et al. 2000). Further, evaluation of DHs generated from transgenic embryos for drought tolerance revealed that the gene *HVAI* which was transferred for improving salt tolerance expressed successfully in DH population across the years of testing. Tolerance to high salt concentration in DH transgenic was much better than DHs without transgene transfer (Chauhan and Khurana 2011).

Genetic modification of haploid cells followed by regeneration enables the direct observation of recessive genes on the phenotype of a non-chimeric plant DH breeding and helps in mutant selection, due to the ease of selection and fixation of mutations and the desired recombinants (DePauw et al. 2011; Wu et al. 2012). The approach has been implemented in wheat by introducing targeted modifications in an exogenous *DsRed* gene and two endogenous wheat genes, including *TaLox2* and TaUbiL1 using microspore culture (Bhowmik et al. 2018). Further, modification of

genes responsible for haploid induction in wheat and maize hybrids for enhancing the number of haploids generated found immense applications in DH breeding (Liu et al. 2019).

7.5 Conclusion and Future Prospects

Haploids as well as doubled haploids are indispensable in various domains of plant breeding including classical plant genetics and cytogenetics, modern molecular genetics, induced mutagenesis, site-directed mutagenesis, genetic transformation research, genome mapping and assessing distant genome relationships, gene dosage effects, analysis of linkages and mechanisms of the genetic control of chromosomal pairing. The DH technology involving distant hybridization offers a rapid mode of truly homozygous line production that helps to expedite wheat breeding programmes swiftly. Among such systems, wheat × maize has been found inefficient in inducing haploids in triticale × wheat and wheat × rye recombinants. The novel invention of wheat × *I. cylindrica* system by H. K. Chaudhary and his associates from Molecular Cytogenetics and Tissue Culture Lab, CSK HPKV, has not only overcome the aforesaid limitation but by generating large number of haploids in various genotypes in bread and durum wheat has also widened the horizons of wheat improvement endeavours. Further, the candidature of wheat × *I. cylindrica* system as the most efficient approach for haploid induction is strengthened by the longevity of its pollen and large area covered by the grass across the globe.

Palampur, a place situated in northwestern Himalayas, provides very congenial environment to the breeding as well as pre-breeding programmes. Here, temperature regime is such that the hybridization experiments can be carried out from mid of March till May end, thus providing a breeder almost 3 months of satisfactory execution of planned crossing experiments naturally. Further, national and international level facilities along with new and innovative protocols developed in this lab can attract scientists from different research platforms with a complete package to accelerate wheat breeding endeavours through speed breeding with utmost precision. The DH technology has been successfully exploited in wheat but can also be extended to other crops like maize, minor millets and pulses where such an efficient system is not available for variety development programmes, pre-breeding programmes and gene mapping studies aimed at commercial-scale breeding. Integration of DH technology with other available biotechnological tools such as marker-assisted selection (MAS), induced mutagenesis and transgenic technologies could ease direct incorporation of genes of interest in candidate crop plants at the haploid level following chromosome doubling and may also effectively accelerate the crop improvement programmes.

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Chapter 8
Acceleration of the Breeding Program for Winter Wheat

Alison M. R. Ferrie and Patricia L. Polowick

8.1 Introduction

8.1.1 The Importance of Winter Wheat

Wheat (*Triticum aestivum* L.) is one of the major crops in the world, relied upon by much of the world's population. In 2017/2018, world wheat production was estimated to be 756.8 million tonnes (FAO—Food Outlook 2018). This crop has the broadest adaptation of all cereals as it is able to grow in many climates due to its tolerance to the cold. The wheat types that have recessive alleles at the *VRN* loci controlling vernalization requirements are termed winter wheat and are usually planted in the fall and harvested in the following year. Winter wheat is grown in North America, Europe, Asia and the Middle East. There are a number of advantages to growing winter wheat as it has the potential to be higher yielding than spring wheat, it matures earlier than spring wheat and therefore can avoid the early frosts and, in addition, growing winter wheat can allow the producer to spread the workload over the growing season. Moreover, winter wheat also acts as a solid ground cover over winter and can take advantage of the early spring moisture.

8.1.2 Winter Wheat Breeding Programs

Similar to those of any crop breeding programs, the goals of winter wheat breeding programs are increased yield, superior end-use quality, durable pest resistance and improved agronomic characteristics. In many programs, the goal is not to increase

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cold tolerance, as that is usually associated with delayed growth and poor yields, but to have the minimal level of winter hardiness for that particular growing area. Because of the vernalization cycle (up to 12 weeks), it can take at least 12 years to develop new winter wheat cultivars (Baenziger and DePauw 2009).

8.1.3 Doubled Haploidy Technology

Doubled haploidy technology has been developed for a number of species. It is the quickest method for developing homozygous lines, which can then be used in a breeding program to shorten the breeding cycle. In addition to speeding up the development of new cultivars by 3–4 years (Ulrich et al. 1984), doubled haploidy methodology allows for easier identification of recessive traits, more efficient mutant selection and easier genetic manipulation. In combination with marker-assisted selection (Wessel and Botes 2014; Humphreys and Knox 2015; Wiśniewska et al. 2019) or gene editing (Bhowmik et al. 2018; Ferrie et al. 2019), doubled haploidy has the potential to increase the efficiency of the breeding process. Recent publications have utilized haploid induction editing technology (HI-Edit) to edit inbred lines in maize (*Zea mays* L.) (Kelliher et al. 2019). Additional experiments were conducted in wheat. The maize pollen parent, transformed with Cas9, was used to pollinate wheat spikes, resulting in edited wheat embryos (Kelliher et al. 2019). Improvements in efficiencies are required to make this system amenable for commercial breeding purposes.

Doubled haploid plants are commonly produced using either in vitro or in vivo methods. In vitro methods include the culture of anthers or microspores (androgenesis) or the culture of unfertilized ovules (gynogenesis). In vivo methods include pollination with inactive (radiated, chemical treated, physically treated) pollen, stimulation of parthenogenesis, intraspecific hybridization and interspecific or intergeneric crosses followed by chromosome elimination. The most efficient method depends on the species; however, some species will not respond to any of these methods; therefore, new methods for haploidization are being explored (reviewed by Kalinowska et al. 2019).

8.1.4 Doubled Haploidy Technology for Winter Wheat

In wheat, haploid plants can be produced using two methods, intergeneric crosses followed by chromosome elimination or culture of anthers or microspores (androgenesis). For the wide crosses method, studies have evaluated different species as the pollen parent, including maize (Laurie and Bennett 1986; O'Donoughue and Bennett 1994; Inagaki 2003), cogon grass (*Imperata cylindrica* (L.) P.Beauv.) (Chaudhary et al. 2005), pearl millet (*Pennisetum glaucum* (L.) R.Br) (Ahmad and Comeau 1990), eastern gammagrass (*Tripsacum dactyloides* (L.)) (Riera-Lizarazu

and Kazi 1993), teosinte (*Zea diploperennis*, *Zea mays* L. ssp. *parviglumis*, *Zea luxurians*, *Zea mays* L. ssp. *mexicana*) (Suenaga et al. 1998), barley (*Hordeum vulgare* L.) (Barclay 1975), sorghum (*Sorghum bicolor* (L.) Moench) (Ohkawa et al. 1992) and Job's tears (*Coix lacryma-jobi* L.) (Mochida and Tsujimoto 2001). The most responsive pollen parent has been maize, although the first Indian DH wheat cultivar was developed using *I. cylindrica* (Chaudhary et al. 2015).

8.1.5 Wheat × Maize Method

There are many reviews outlining the steps required for producing DH plants using the wheat × maize method (Chaudhary et al. 2015; Humphreys and Knox 2015; Srivastava and Bains 2018; Patial et al. 2019). This method is very labour intensive and requires growth space for both wheat and maize plants. Protocols do vary among laboratories, but generally wheat plants are emasculated and then pollinated by maize pollen. After 2 days, 2,4-D (2,4-dichlorophenoxyacetic acid) is injected into wheat florets to enhance embryo development (Suenaga and Nakajima 1989). After an additional 14 days, embryo rescue is required to recover haploid plants. The frequency of embryo development can vary. Four embryos per wheat spike with a 50–70% rate of regeneration from embryos to plants (Inagaki 2003) has been reported. The maize pollination method generates haploid plants; therefore, chromosome doubling by colchicine treatment is required. This can delay the production of a doubled haploid plant. Although this method is inefficient compared to haploid production in other species, over 50 wheat cultivars have been developed in Canada and other countries (Humphreys and Knox 2015 Tables 8.1 and 8.2).

8.1.6 Androgenesis

In 1973, the first successful regeneration of a DH plant from wheat anthers was achieved (Ouyang et al. 1973; Picard and De Buyser 1973), and since then there have been many improvements in anther culture and isolated microspore culture methods (Soriano et al. 2007, 2008; Cistué et al. 2009). Anther culture involves plating the intact anther on solid or liquid media, whereas microspore culture involves isolating the microspores from the anther and culturing only the microspores in liquid media. When given the inductive conditions for switching from gametophytic to sporophytic development, the embryogenic immature pollen grains will develop into calli or embryos. These structures can then be regenerated to plants and treated with a chromosome-doubling agent to generate fertile doubled haploid plants. Most of the early work has been with anther culture, and there continues to be improvements made to the method to increase efficiency; however, isolated microspore culture is the preferred method (Ferrie and Caswell 2011). The main advantage of microspore culture over anther culture is the lack of somatic diploid tissue, i.e., anther wall, which could under certain conditions produce calli and/or embryogenic

Table 8.1 Registered Canadian winter wheat cultivars developed using doubled haploid breeding (modified from Humphreys and Knox 2015)

Year	Cultivar	Method	Breeding institute[a]	Reference
2001	Warthog	Maize pollination	Hyland Seeds—DAS	
2002	FT Wonder	Maize pollination	AAFC-ECORC	
2005	Ashley	Maize pollination	AAFC-ECORC	
2005	Emmit srw	Maize pollination	Hyland Seeds—DAS	
2005	FT Action	Maize pollination	AAFC-ECORC	
2007	Ava sww	Maize pollination	Hyland Seeds—DAS	
2007	Carnaval	Maize pollination	Hyland Seeds—DAS	
2007	Wentworth hrw	Maize pollination	Hyland Seeds—DAS	
2008	Accipiter	Maize pollination	University of Saskatchewan	Fowler (2011)
2008	Broadview	Maize pollination	AAFC-LRC	
2008	Peregrine	Maize pollination	University of Saskatchewan	Fowler (2010)
2009	HY116-SRW	Maize pollination	Hyland Seeds—DAS	
2009	HY124-SRW	Maize pollination	Hyland Seeds—DAS	
2009	Sunrise	Maize pollination	University of Saskatchewan	Fowler (2012)
2010	Flourish	Maize pollination	AAFC-LRC	Graf et al. (2012)
2011	Emerson	Maize pollination	AAFC-LRC	Graf et al. (2013)
2011	OAC Emmy	Maize pollination	University of Guelph	Tamburic-Ilinicic and Smid (2013a)
2011	Pintail	Maize pollination	AARD-FCDC	Salmon et al. (2015)
2011	Swainson	Maize pollination	University of Saskatchewan	Fowler (2013)
2011	HY271-SRW	Maize pollination	Hyland Seeds—DAS	
2012	HY300-HRW	Maize pollination	Hyland Seeds—DAS	
2012	OAC Flight	Maize pollination	University of Guelph	Tamburic-Ilinicic and Smid (2013b)

(continued)

Table 8.1 (continued)

Year	Cultivar	Method	Breeding institute[a]	Reference
2013	CDC Chase	Maize pollination	University of Saskatchewan	Fowler (2014)
2013	HY301-HRW	Maize pollination	Hyland Seeds—DAS	
2015	AAC Elevate	Maize pollination	AAFC-LRC	Graf et al. (2015)
2015	Marker	Maize pollination	University of Guelph	Tamburic-Ilinicic and Smid (2015a)
2015	UGRC Ring	Maize pollination	University of Guelph	Tamburic-Ilinicic and Smid (2015b)
2018	AAC Icefield	Maize pollination	AAFC-LRC	Graf et al. (2018)

[a]Breeding institutions: DAS, Dow AgroSciences; AAFC-ECORC, Agriculture & Agri-Food Canada–Eastern Cereal and Oilseed Research Centre; AAFC-LRC, Agriculture & Agri-Food Canada–Lethbridge Research Centre; AARD-FCDC, Alberta Agriculture and Rural Development-Field Crop Development Centre

Table 8.2 Examples of winter wheat doubled-haploid cultivars developed in other countries

Year	Country	Cultivar	Method	Reference
1983	China	Jinghua No. 1	Anther culture	Hu et al. (1983)
1987	France	Florin	Anther culture	De Buyser et al. (1987)
1992	Hungary	GK Délibáb	Anther culture	Pauk et al. (1995)
2001	Sweden	SW Agaton	Anther culture	Tuvesson et al. (2003)
2005	Romania	Glosa	Maize pollination	Săulescu et al. (2012)
2005	Romania	Grandur	Maize pollination	Giura (2007)
2010	Romania	Litera	Maize pollination	Săulescu et al. (2012)
2011	Romania	Miranda	Maize pollination	Săulescu et al. (2012)
2015	Chile	Konde INIA	Maize pollination	Jobet et al. (2015)
2018	USA	Avery	Maize pollination	Haley et al. (2018a)
2018	USA	Langin	Maize pollination	Haley et al. (2018b)

structures. For both androgenic methods, there are a number of factors affecting embryogenesis, including genotype, donor plant growth conditions, stage of microspore development, composition of the culture medium and environmental conditions during culture. The frequency of embryo production will depend on whether or not these conditions are optimal. Over the years, several factors (Table 8.3) have been evaluated to enhance anther culture and isolated microspore culture response. Genotype plays a major role in embryogenic response, and therefore the goal of any androgenic doubled haploidy method is to have a genotypic independent protocol. Much of the focus has been on spring wheat cultivars, which appear to be more responsive than winter wheat genotypes.

The quality of the donor plants is important to successful androgenesis. Healthy, vigorously growing, disease- and stress-free plants are critical. Temperature,

Table 8.3 Factors influencing androgenic response in wheat

Factors		References
Donor plant conditions	Photoperiod	Jones and Petolino (1987)
	Light intensity	Bjornstad et al. (1989)
	Temperature	Jones and Petolino (1987), Simmonds (1989)
Pre-treatment	Temperature	Datta and Wenzel (1987), Indrianto et al. (1999)
	Mannitol	Kasha et al. (2003), Shirdelmoghanloo et al. (2009), Santra et al. (2012)
	Addition of cupric sulfate	Brew-Appiah et al. (2013)
Developmental stage of the microspore	Mid-late uninucleate	Ouyang et al. (1973), Picard and De Buyser (1973), He and Ouyang (1984), Liu et al. (2002)
Media composition	Carbohydrate source	Last and Brettell (1990), Orshinsky et al. (1990)
	Ovary co-culture	Zheng et al. (2002)
	Dimethyl tyrosine conjugated peptide SS-31	Sinha and Eudes (2015)
	Gum Arabic, larcoll	Letarte et al. (2006)
	Casein hydrolysate	Datta and Wenzel (1987)
	Ficoll	Jiang et al. (2017)
	Phytosulfokine alpha	Asif et al. (2014)
	Antioxidants	Asif et al. (2013a)
	Cefotaxime	Asif et al. (2013b)
	Polyamines	Redha and Suleman (2013)
	Cupric sulfate	Grauda et al. (2014)
	2-Hydroxynicotinic acid	Zheng et al. (2001)
	Amino acids	Pauk et al. (2003)
	Histone deacetylase inhibitors (trichostatin BIX-01294, scriptaid, sodium butyrate)	Kathiria et al. (2016), Jiang et al. (2017), Wang et al. (2019)

photoperiod and light intensity can all play a role in the embryogenic response of wheat anther and microspore cultures (Jones and Petolino 1987; Bjornstad et al. 1989; Simmonds 1989). Identification of the microspores at the developmental stage that will be best for embryogenic response is essential. In most crops, and wheat is no exception, the mid-late uninucleate stage of development is optimal (Ouyang et al. 1973; Picard and De Buyser 1973; Liu et al. 2002). Unlike in some crops where a pre-treatment of the inflorescence is not necessary for the switch from gametophytic to sporophytic development, in wheat a cold temperature, mannitol or the addition of cupric sulfate have all been employed to enhance embryogenesis (Datta and Wenzel 1987; Indrianto et al. 1999; Kasha et al. 2003; Shirdelmoghanloo et al. 2009; Santra et al. 2012; Brew-Appiah et al. 2013).

The composition of the media is also a major factor influencing microspore embryogenesis and has been the focus of many research projects and manuscripts. Early studies looked at carbohydrate source and concentration (Last and Brettell 1990; Orshinsky et al. 1990), while more recent studies have evaluated chemical enhancers (Zheng et al. 2001), arabinogalactan proteins (Letarte et al. 2006), n-butanol (Soriano et al. 2008), antioxidants (Asif et al. 2013a), antibiotics (Asif et al. 2013b), histone deacetylase inhibitors (HDACi) (Jiang et al. 2017; Wang et al. 2019) and other epigenetic compounds (Wang et al. 2019). The HDACi compound Trichostatin (TSA) at nanomolar concentrations (0.008–0.010 μM) was beneficial to enhance microspore embryogenesis in winter wheat. TSA in the culture medium, along with a cold pre-treatment of 28 days and a 23% maltose gradient, induced the production of up to 1981 embryos/spike (Wang et al. 2019).

Over the years, there have been significant improvements in the embryogenic response of wheat microspore cultures; however, there are still a few issues that need to be resolved to increase the efficiency of the protocol for breeding purposes. Albinism is a major problem in many cereal anther/microspore doubled haploidy protocols, and wheat is no exception. Some genotypes produce more albinos than others (Liu et al. 2002; Barnabás 2003; Tuvesson et al. 2003; Zheng et al. 2003). While improvements have been made, further manipulation of the culture media and culture conditions is required to decrease the number of albino plants.

We have developed wheat doubled haploidy protocols for spring wheat and winter wheat. There are slight differences between the two protocols, but this is mainly with the length of pre-treatment (Ferrie et al. 2019; Wang et al. 2019). Protocols have been validated with a range of cultivars and breeding material from different organizations, as well as in different laboratories (Wang et al. 2019). For winter wheat, embryogenic frequency ranged from 33 to 1981 embryos per spike, which resulted in 1 to 276 green plants/spike (Wang et al. 2019). The protocol (Ferrie et al. 2019; Wang et al. 2019) that we use is outlined below.

8.1.6.1 Winter Wheat: Growing Donor Plants

- Seeds are planted into pots and kept in growth cabinets set at 20/18 °C, 18 h photoperiod, and 350–420 μmol/m^2/s light intensity.
- After 2 weeks, the pots are transferred to a 3–4 °C, 12 h photoperiod, 150 μmol/m^2/s setting for a 9-week vernalization treatment.
- After vernalization, the pots are transferred to a 15/12 °C setting, with an 18 h photoperiod at 400–420 μmol/m^2/s.

8.1.6.2 Collecting Spikes for Microspore Isolations

- Before spikes can be collected for experiments, the correct microspore stage has to be determined. The mid-late uninucleate stage is the optimal stage, based on our plant growth conditions (Fig. 8.1a).

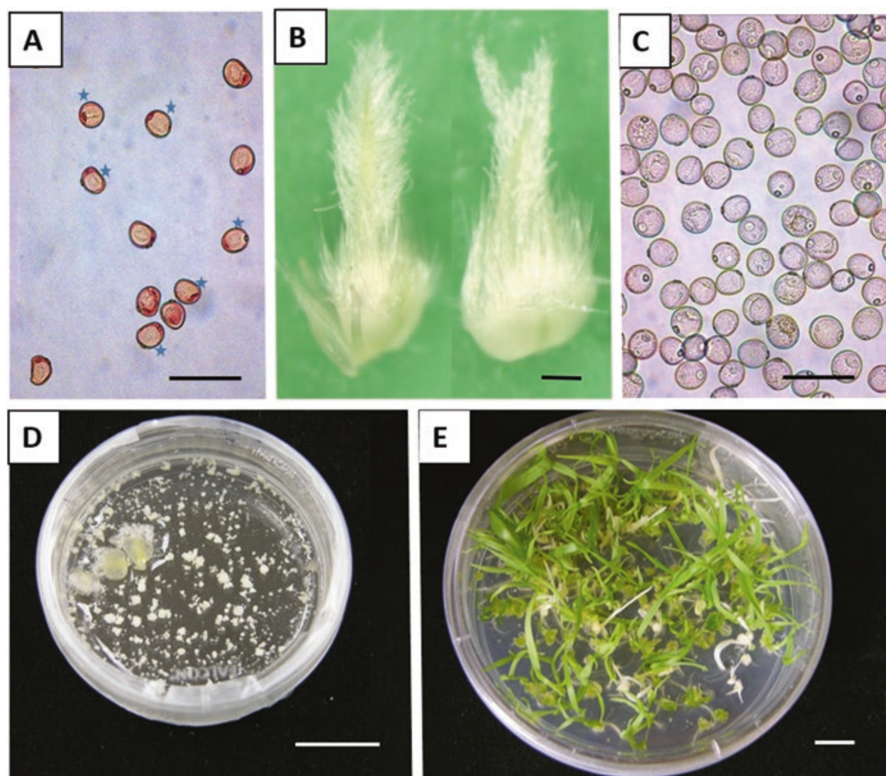

Fig. 8.1 Development of doubled haploid plants in winter wheat. (**A**) Acetocarmine stain of wheat microspores at the mid to late uninucleate stage; asterisk indicates optimal stage. Scale bar, 100 μm. (**B**) Wheat ovary developmental stages; elongated (left side), cleft (right side). Scale bar, 250 μm. (**C**) Wheat microspores at the time of isolation. Scale bar, 100 μm. (**D**) Wheat microspore derived embryo development after 28 days in culture. Scale bar, 1 cm. (**e**) Haploid/doubled haploid plants. Scale bar, 1 cm

- Collect spikes and trim off leaves, leaving 2–3 cm of the flag leaf. Place spikes in a (250-mL) flask containing about 200 mL of cold water.
- Store spikes at 4 °C for 28–30 days before experimentation.
- Use ten spikes per experiment, depending on genotype.

8.1.6.3 Collecting and Sterilization of Spikes for Ovaries

- Staging the ovary heads prior to the day of experimentation is recommended. Ovaries at the elongated/cleft stage are desired (Fig. 8.1B).
- Sterilize ovary donor spikes in a 500-mL glass bottle with approximately 500 mL of 20% bleach and Tween 80 (3 drops/500 mL) for 5 min. Rinse four times with 500 mL of cold sterile water. Store the sterilized heads in the bottle at 4 °C until needed.

8.1.6.4 Sterilization of Spikes for Microspore Isolation

- Sterilize spikes in a 500-mL glass bottle with 500 mL of 10% bleach with Tween 80 (3 drops/500 mL) for 3 min. Rinse four times with 500 mL of cold sterile water.

8.1.6.5 Microspore Isolation

- Use cold equipment (4 °C) and media. The sterile spikes for microspore isolation are kept on ice.
- Remove each spikelet from the rachis and transfer to a medium-sized blender cup that contains FHG-2 extraction solution (Table 8.4).
- Blend the spikelets on low speed twice for 10 s and pour the blended material through a 90-μm filter funnel into a sterile bottle. Rinse the blender cup with FHG-2 solution and pour through the filter.
- Pour the filtrate into 50-mL centrifuge tubes and centrifuge at 1000 rpm 4 °C for 5 min.
- Decant, then add FHG-2 solution and centrifuge at 1000 rpm 4 °C for 5 min.
- Decant and resuspend pellet in 5 mL of cold NPB-99 medium with glutathione, larcoll and cefotaxime (NPB99 + GLC). Pour microspore suspension into a 15-mL tube. Rinse the 50-mL tube with an additional 5-mL cold NPB99 + GLC and pour into the 15-mL tube. Repeat to a final volume of 15 mL in the 15-mL tube.
- Centrifuge at 1000 rpm 4 °C for 5 min.
- Decant and add 10 mL of cold 23% maltose in water. Gently re-suspend the microspores. Layer 1 mL of cold NPB99 + GLC over the maltose solution. Centrifuge at 1000 rpm 4 °C for 13 min. A distinct band of microspores will form at the interface. Remove the band of microspores and transfer to a clean sterile a 15-mL tube. Bring microspores to 6 mL volume with cold NPB99 + GLC and then to 15 mL volume with cold 0.3-M mannitol. Invert to mix.
- Centrifuge at 1000 rpm 4 °C for 5 min. Decant.
- Add NPB99 + GLC and count microspores with a haemocytometer (Fig. 8.1C).
- Add NPB99 + GLC to the tube to yield a density of 60,000 microspores per mL. Plate 1.5 mL of microspore suspension per 35 × 10 mm plate.
- TSA is added to the Petri plates at a concentration of 0.008–0.01 μM. We use stock concentrations in dimethyl sulfoxide (DMSO) at 50 μM and 5 μM TSA.

8.1.6.6 Addition of Ovaries

- Isolate ovaries from the sterile ovary spikes selecting elongated/cleft ovaries.
- Place three ovaries into each plate of microspores.
- Wrap with Parafilm® and place plates inside a large Petri dish (150 × 25 mm) with an open 60 × 15 mm Petri dish filled with water in the centre.
- Incubate in the dark at 28 °C for 4–5 weeks. Do not disturb.

Table 8.4 Composition of media used in wheat microspore culture and plantlet regeneration

Media components (mg/L)	FHG-2 solution	NPB-99$^+$ medium	B5-5 regeneration
KNO_3	1900.00	1415.0	2500.0
$(NH_4)NO_3$	165.00		
$(NH_4)_2SO_4$		232.0	134.0
KH_2PO_4	170.00	200.0	
$NaH_2PO_4 \cdot H_2O$			150.0
$CaCl_2 \cdot 2H_2O$	440.00	83.0	150.0
$MgSO_4 \cdot 7H_2O$	370.00	93.0	250.0
$FeSO_4 \cdot 7H_2O$	27.85	27.8	27.85
Na_2EDTA	37.25	37.3	37.25
$MES \cdot H_2O$	1950.00		1000.0
KI		0.4	0.75
$MnSO_4 \cdot H_2O$		5.0	10.0
H_3BO_3		5.0	3.0
$ZnSO_4 \cdot 7H_2O$		5.0	2.0
$CoCl_2 \cdot 6H_2O$		0.0125	0.025
$CuSO_4 \cdot 5H_2O$		0.0125	0.025
$Na_2MoO_4 \cdot 2H_2O$		0.0125	0.25
Thiamine HCl		5.0	10.0
Pyridoxine HCl		0.5	1.0
Nicotinic acid		0.5	1.0
Myo-inositol		50.0	100.0
Glutamine		500.0	
Glutathione		0.61	
Larcoll		40.0	
Sucrose			20,000.0
Maltose		90,000.0	
Mannitol	72,870.00		
2,4-D		0.2	
Kinetin		0.2	1.0
PAA		1.0	
IAA			1.0
Cefotaxime		100.0	
pH (final)	6.5	6.5	5.8
Difco agar			7000.0

8.1.6.7 Plating Embryoids

- After 28–35 days incubation at 28 °C, count the embryoids (Fig. 8.1D).
- Pour the embryoids onto solid B5-5 medium. If the embryoid count is very high, distribute the embryoids over several plates such that there are approximately 100–200 embryoids per plate (100 × 25 mm plates).

- Seal plates with Parafilm® and place at 22 °C, with a 16-h photoperiod and 100-µmol/m²/s light intensity. Shoots should start developing within 1–2 weeks (Fig. 8.1E).

8.1.6.8 Plantlet Development

- After an additional 2–3 weeks, green shoots can be transferred to Magenta boxes or glass jars containing B5-5 medium for continued development.
- The plantlets can be checked for ploidy to determine if a chromosome-doubling agent is required. Doubled haploid plants can be transferred directly to soil. If no spontaneous doubling took place, plantlets can be treated with colchicine, trifluralin or other chromosome-doubling agents.

8.1.7 Doubled Haploids in a Breeding Program

The benefits of utilizing DH in a plant breeding program include the savings in time and resources to generate a homozygous line. This time savings can be 3–5 years, depending on the species (Ulrich et al. 1984; Barkley and Chumley 2012). An example of a comparison of a doubled haploidy breeding program and one using conventional plant breeding is shown in Fig. 8.2. There is increased efficiency in selection and the ability to identify recessive traits as there is no dominance factor (Forster and Thomas 2010).

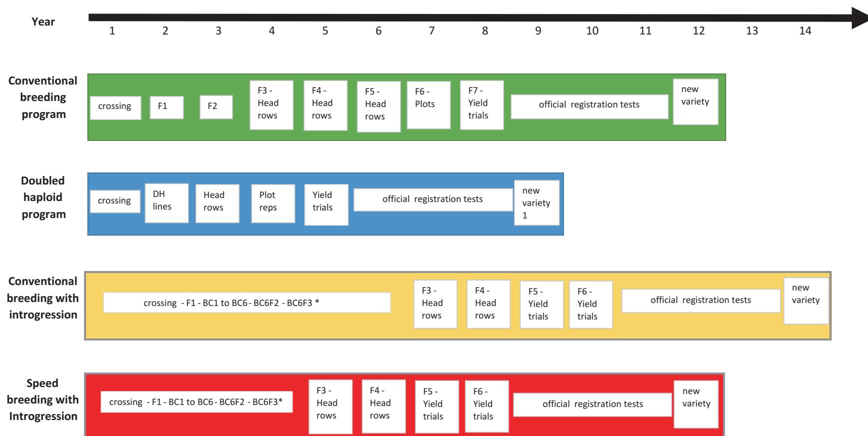

Fig. 8.2 Timeline comparisons of conventional breeding programs for crosses and introgression with the doubled haploidy and speed breeding processes

Several studies have compared the DH methodology and conventional pedigree breeding methods (Guzy-Wróbelska and Szarejko 2003; Săulescu et al. 2012; Li et al. 2013; Tadesse et al. 2013). Guzy-Wróbelska and Szarejko (2003) compared plants derived from anther culture, maize pollination and single-seed descent under field conditions and found that the plants were similar in agronomic response. In a follow-up study (Guzy-Wróbelska et al. 2007), the authors found that the recombination frequency in anther culture-derived lines was higher than in maize pollination-derived lines. Another study evaluated grain yield between DH lines (produced via maize pollination) and those from pedigree selection (Inagaki 1998). They determined that grain yields were the same when the parental lines were closely related; however, when the parental lines were less related, the DH lines had lower grain yield than the lines derived from single-seed descent or pedigree selection. Therefore, it is important to have large population sizes, 100–700 DH lines, to evaluate in order to increase the probability of selecting desired recombinants. Other studies have shown that the CIMMYT conventional breeding methods were more efficient genetically and economically than their DH (maize pollination) method (Li et al. 2013). Utilizing literature and discussions with wheat breeders and industry, Barkley and Chumley (2012) developed economic models to compare the use of DH in wheat breeding programs. They concluded that large economic gains would be realized with the use of DH technology.

Most of the wheat DH cultivars have been produced via anther culture or wheat × maize crosses (Tables 8.1 and 8.2) (Humphreys and Knox 2015; Patial et al. 2019). The first anther culture cultivars in wheat included Jinghua No. 1 (Hu et al. 1986), Florin (De Buyser et al. 1987) and GK Délibáb (Pauk et al. 1995). Both Jinghua No. 1 and GK Délibáb are winter wheat cultivars. In Canada, approximately 50 DH wheat cultivars have been developed, 28 of which are winter wheat that were developed through the wheat × maize cross method (Table 8.1). Doubled haploid wheat cultivars have also been registered in Europe, North America and South America (Table 8.2). Obviously, this list is not complete as plant breeders do not have to provide the methods in which they developed the new cultivars.

8.1.8 Speed Breeding

Speed breeding, also known as accelerated breeding, is another tool available to hasten crop breeding. The concept was first tested by NASA, along with Utah State University, in an effort to assess food production under the constant light on space stations, starting with wheat. A group of researchers, led by University of Queensland, expanded both the range of crop species and the investigation of additional growth parameters, including the harvest of immature seeds. They have tested conditions for a number of crops, including spring, durum (*Triticum durum* Desf.) and winter wheat, barley, chickpea (*Cicer arietinum* L.), pea (*Pisum sativum* L.) and canola (*Brassica napus* L.), evaluating day length, light quality and intensity, and temperature (Ghosh et al. 2018; Watson et al. 2018). There has also been a directed

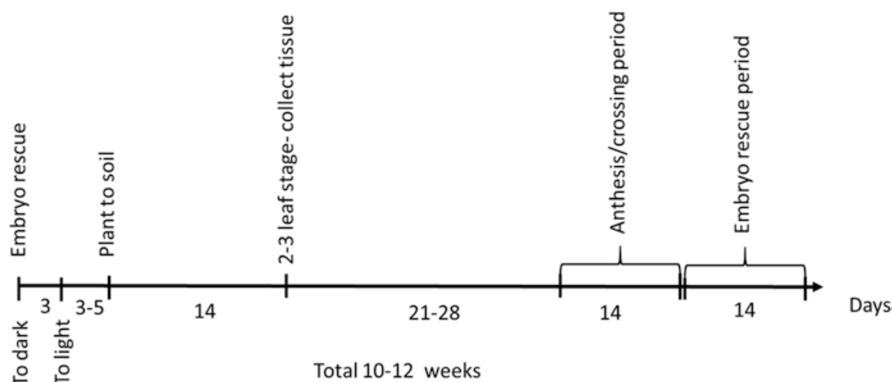

Fig. 8.3 Timeline for the accelerated crossing of spring wheat

focus on hastening the life cycle of legumes, with an emphasis on early flowering and the germination of immature seeds (Croser et al. 2016; Ribalta et al. 2017). The systems developed resulted in a savings of 47–54 days per generation and the production of up to five generations of *Lupinus angustifolius* L. (lupin) or six generations of pea. Similarly, six (Watson et al. 2018) to eight (Zheng et al. 2013) generations of spring wheat or nine generations of barley (Zheng et al. 2013) could be cycled in 1 year, the latter study using embryo rescue to preclude seed maturation.

We have recently used this technology to speed up the introduction of genes of interest for a trait influenced by three alleles, using both spring and winter wheat, as assessed by genotyping at each generation. In addition to the long photoperiod, in this case 24-h light, and embryo rescue, stresses imposed on the plants also included small pot size, reduced watering and increased temperature.

Under our conditions, depending on genotype, a spring wheat life cycle from embryo rescue to embryo rescue was 70–84 days (10–12 weeks) (Fig. 8.3), including crossing. The longer times also reflect instances where secondary tillers were encouraged to maximize the number of embryos recovered and to match the timing of maturation of the donors and recipient material. There was some variability in timing due to plant genotype but great uniformity within genotypes. This is comparable to the 66 days of speed breeding conditions with a single tiller (Hickey et al. 2019) or 77 days from sowing to harvest when combined with phenotyping (Alahmad et al. 2018). This is a significant reduction from the 113 day generation time reported for spring wheat maturation under field or normal greenhouse conditions (Hickey et al. 2019) or in a range of 3–4 months (90–120 days) in the greenhouse (http://www.wheat-training.com/wp-content/uploads/Wheat_growth/pdfs/Wheat_development_pdf.pdf). An example of a comparison of a breeding program involving introgression using accelerated breeding conditions and one using conventional plant growth conditions is shown in Fig. 8.2 and suggests a potential savings of 2 years.

The winter wheat activity involved six recurrent parents and one donor parent selected for crossing under rapid generation conditions with adjustments made for

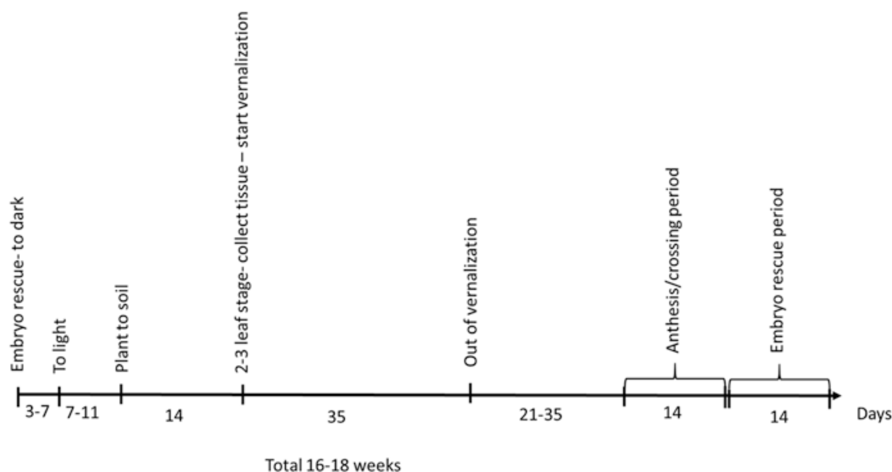

Fig. 8.4 Timeline for the accelerated crossing of winter wheat

vernalization required by the winter wheat condition. All recurrent parents were crossed with a donor parent to transfer three genes, with subsequent rounds of back-crossing to the original female recipient line. Under the speed breeding conditions listed and using the protocol below, the generation time from embryo rescue to embryo rescue, including vernalization, was 16–18 weeks (Fig. 8.4). As with the spring wheat, this is a significant reduction from the 6–8 months (24–32 weeks) reported for winter wheat under greenhouse conditions (http://www.wheat-training.com/wp-content/uploads/Wheat_growth/pdfs/Wheat_development_pdf.pdf).

The goal of 40 rescued embryos per crossing scheme for each crossing generation was generally exceeded. Especially with later generations, significantly more (100s) were generated to ensure that the next generation in the scheme was not lost and as many pollen donors as possible were recovered. Despite the calculated probabilities of offspring containing all three desired alleles, there were occasions where hundreds of plants were required to be screened in order to identify a few positive for all three alleles of interest.

Embryo rescue—the rate of success of embryo rescue was lower with winter wheat than with spring wheat, where more than 98% survived. Approximately 10% of embryos were lost in the rescue process. While this represents a 90% germination rate, a further loss of 10–15% during vernalization yielded a success rate of 75%. In comparison, 80% viability was reported after harvesting and direct planting of immature embryos from spring wheat 14 days after anthesis (Watson et al. 2018). Also, a success rate ranging from 85% to 95%, with variability between genotypes, was observed when planted directly after an early harvest 18 days post heading under 22-h photoperiods in LED-supplemented glasshouses (Watson et al. 2018, suppl data). While direct planting does represent a savings of labour, the present study focused on the recovery of as many donor parent lines as possible through embryo rescue.

Vernalization—a preliminary testing of vernalization duration, in the absence of crossing, helped to set 5 weeks as an adequate vernalization period; this resulted in a period of 80–90 days from sowing to anthesis with variability between the genotypes tested. The timing to anthesis was longer with plated rescued embryos transferred directly into vernalization but shorter when vernalization took place at the two- to three- leaf stage. The difference reflected the time to reach that developmental stage. Ghosh et al. (2018; suppl. data) listed the mean number of days from sowing to flowering of winter wheat at approximately 105 days under a 22-h photoperiod in a glasshouse with an 8-week vernalization period.

With respect to the vernalization of germinated embryos, either from rescue or seed versus at the two- to three-leaf stage, we cannot overemphasize the value of the latter. The viability of the plantlets increased as more plantlets survived; transplantation to soil after vernalization created a shock, and some plants failed to mature. Vernalization of embryos on a plate more often resulted in contamination and the eventual loss of larger numbers of samples/donors. It was also noted that vernalizing two to three leaf donors is convenient with respect to genotyping as 5 weeks of vernalization leaves a generous timing window; there is less of a chance of needing to cross blindly before genotyping is completed. The risk of losing a line does not outweigh the space saving that the vernalizing of embryo rescue plates gives, providing there is an available growth chamber with the capability of maintaining vernalization temperatures.

Lighting—specialized lighting, especially that enriched with far-red wavelengths, has been increasing in use in recent years for a variety of plant and in vitro research, including accelerated breeding. This has been applied by extending the photoperiod and supplementing with LED lighting glasshouses and in benchtop cabinets, as described by Ghosh et al. (2018), as well as in controlled-condition chambers. Specifically for grain legumes (peas, lupins), the promotion of earlier flowering was successful with a decreased red:far red (R:FR) wavelength ratio with consideration of the total photon load in the FR range (Croser et al. 2016; Bennett et al. 2017). The variability reflects the differential requirements of specific species, as well as access to controlled growth cabinets. In our study, Valoya G2 lighting, enhanced in red and far-red light but reduced in PAR (photosynthetically active radiance) was tested; it is designed to enhance vernalization and rooting (https://www.valoya.com/spectra/). When used during the vernalization period, it was found to improve the quality of plantlets put into vernalization at the two- to three- leaf stage, as well as seedlings arising from vernalized embryos on plates (Fig. 8.5). This may be a reflection of the light experience during fall-winter conditions of vernalizing wheat in the field. In order to hasten development, the current project included continuous (24 h) light with a 20/4-h 22 °C/18 °C temperature regime for all growth stages, aside from vernalization. This is warmer than the usual 20 °C/18 °C condition normally used in- house for other studies, including double haploid donor plant conditions. While the higher temperature hastens growth and maturation, it is important to avoid high temperatures at sensitive stages such as meiosis (Hickey et al. 2019). A 22-h photoperiod rather than continuous light was shown to improve plant health (Watson et al. 2018; Ghosh et al. 2018). For legumes, a 20-h

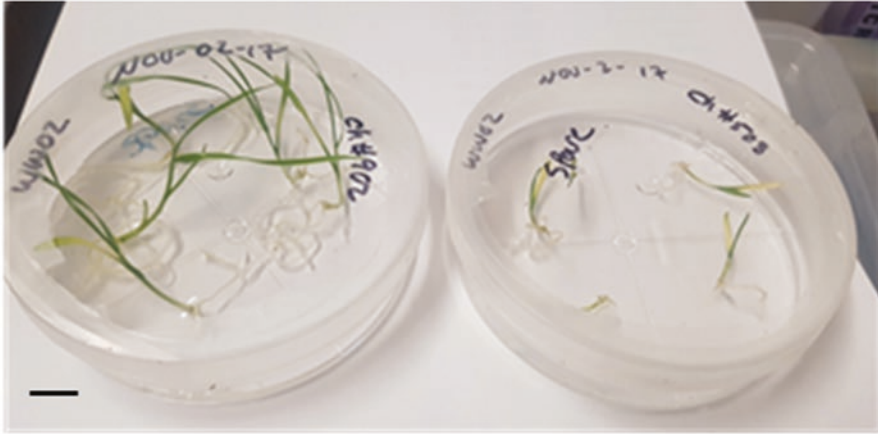

Fig. 8.5 Plantlets from embryo rescue after vernalization under normal (right) and Valoya G2 (left) lighting. Scale bar, 1 cm

photoperiod was used during studies to reduce generation cycle time (Croser et al. 2016). In the present study, the height and vigor of the donor plants were reduced after four to five generations under continuous light. This observation advocates for an adjustment in future endeavours to adjust to a similar 22-h photoperiod.

Additional stressors—in addition to continuous lighting and embryo rescue, our study used smaller pot size for the donor plants (2.5 cm cells in 72-cell propagation trays) to limit root expansion, as well as watering for donor plants for additional stress to hasten flowering and maturation. This approach was also followed for wheat and barley (Zheng et al. 2013), resulting in an increased number of generations achieved in 1 year (up to eight), but yield was dramatically reduced to two to three seeds. With higher density plantings of 300–1000 plants per square metre, the longer photoperiod conditions were shown to hasten maturity in single-seed-descent programs (Ghosh et al. 2018). In our case, in the absence of crossing, the average number of seeds still remained greater than 15 but was more variable with crossing. Hickey et al. (2019) also listed a number of potential improvements that could be added to hasten the generation time, including an elevated concentration of CO_2, hydroponics and breaking of seed dormancy with plant growth regulators that promote germination. Hydroponics and the addition of flurprimidol to reduce plant height have been used to speed up the generation time in peas (Mobini and Warkentin 2016).

Aside from the vernalization period, embryo rescue and accelerated breeding conditions sped up all aspects of winter wheat growth. The timeline, while variable between genotypes, is summarized in Fig. 8.5 and shows a generation time, from embryo rescue to embryo rescue, including crossing, of 16–18 weeks under the stressed conditions of the speed breeding program, with embryo rescue and vernalization at the two- to three-leaf stage. This is slightly longer than the timing achieved without crossing but still represents a reduction in the generation time of winter

wheat, in comparison to the 24–32 weeks under controlled conditions in house at NRC-Saskatoon, albeit for different genotypes. With the completion of backcrossing and selfing to identify lines that are homozygous for the three genes of interest, several spring lines are currently in field trials to determine the efficacy of the introgressed genes.

Speed breeding has been utilized extensively and combined with a variety of selection methods to introduce desirable traits into wheat (Li et al. 2018). As noted by Hickey et al. (2019), this system can be used with all current genotypic and phenotypic selection systems to help plant breeders deal with the changing environment and evolving plant disease. It has been paired with multi-trait phenotyping of quantitative traits in barley (Hickey et al. 2017), as well as root traits, disease resistance and plant height in durum wheat (Alahmad et al. 2018). The first spring wheat variety benefiting from a speed breeding approach was released in Australia in 2017 (Hickey et al. 2019). This involved introgression of genes relating to grain dormancy. In studies of adult plant resistance (APR), it was determined that the degree of resistance to rust of spring wheat lines was consistent between plants grown under normal and speed breeding conditions (N. Rajagopalan, pers. comm.), suggesting that this is a valid means to hasten evaluation (of disease resistance) in new lines. Similarly, there have been studies on utilizing speed breeding for phenotyping resistance to stripe rust (Hickey et al. 2011), leaf rust (Riaz et al. 2016) and *Fusarium* head blight (FHB) (Watson et al. 2018). A heavy focus on disease resistance reflects the urgency for breeders to stay ahead of the constantly evolving threats to the crops.

Early selection resulting from accelerated breeding could reduce the time required to introduce desired traits into elite breeding lines. The protocol has been paired with single-seed-descent protocols for wheat and barley (Ghosh et al. 2018) and genetic transformation of barley (Watson et al. 2018). It has been used in tandem with hydroponics to assess boron resistance in pea (Bennett et al. 2017); this would suggest an application for screening for salt tolerance. In all cases, this approach has the potential to promote the genetic gain targets required for a secure future (Li et al. 2018).

The accelerated methodologies are an option for laboratories that do not have tissue culture facilities or DH capabilities and are the best option where an equal contribution from the two parents in a cross is not the desired outcome. At the same time, it would be possible to combine the approach with DH with respect to the growth and maturation of doubled haploid plants.

8.1.8.1 Winter Wheat Speed Breeding Protocol

Growth of Recurrent Parents

- Germinate seed on moistened sterile filter paper for 3 days in the dark at room temperature. Seal the plates with Parafilm®. If vernalizing on filter paper, also surface sterilize the seed. Place the plates with germinated seed into a tissue culture chamber (constant 24 °C, 16 h photoperiod) for 2 days.

- It is preferable to grow the seedlings to two- to three-leaf stage prior to vernalization (see note below). Transplant into Sunshine Mix #4, amended with four pellets of slow release 14-13-13 fertilizer, in 2.5 cm cells of 72-cell propagation trays, and place them into a growth chamber with accelerated conditions (24 h light, with a temperature regime of 16 h/8 h 22 °C/18 °C, lighting at 650 μmol/m^2/s), for ~1 week or until such time as they are at the two- to three-leaf stage. During the growth of the seedlings, use vented domed lids to maintain humidity around the transplanted seedlings until they almost touch the lid. During this time, watering is not generally required.
- Place the recurrent lines into a vernalization chamber (Valoya G2 lighting at 80 μmol/m^2/s with an 8-h photoperiod, at a constant temperature of 4 °C) for 5 weeks.
- Start planting recurrent parents at the same time that the embryo rescues of the winter crosses begin and continue for several subsequent weeks (at least 5) to ensure a good coverage for crossing.
- While the plants are in vernalization, check every 2 days for watering. They will not need much water as they are not growing rapidly and the cooler temperature minimizes evaporation. Supply 1 g/4 L 20:20:20 fertilizer/water every 2 weeks.
- At the end of the 5-week vernalization period, place the plants for 1–2 weeks into a growth chamber with accelerated conditions (24 h light, with a temperature regime of 16 h/8 h 22 °C/18 °C lighting at 650 μmol/m^2/s) to allow acclimatization to new temperature and lighting and to allow roots to establish. Then transplant the recurrent plants individually into 9-cm pots. Fertilize twice weekly with 20:20:20 fertilizer.

Embryo Rescue of Donors (Protocol Modified from Zheng et al. 2013)

- Embryos from embryo rescue are ready to be excised as early as 14 DPA (days post anthesis/crossing) with a window of approximately 14–18 days, depending on the maturity of the developing seed. Follow the embryo rescue protocol below.
- Medium (modified from https://www.k-state.edu/wgrc/electronic_lab/embryo-rescue_medium.html):
  - Murashige and Skoog Basal Salts with minimal organics (4.0 g) Sigma M6899 (Linsmaier and Skoog 1965).
  - $NaH_2PO_4H_2O$ (final concentration 170 mg/L).
  - I-inositol (final concentration 100 mg/L).
  - Thiamine (final concentration 20 mg/L).
  - L-tyrosine, L-arginine, glycine (final concentration of each 10 mg/L).
  - Sucrose (6%).
  - pH 5.7.
  - Phytagel (0.52%).
  - 25 × 100 mm Petri dishes to allow for the growth of the coleoptile.

- Prior to use, the plates may be placed in the hood with the lids at an angle to remove any condensation.
- Developing grains 14 DAP or older are surface sterilized with 1% sodium hypochlorite solution for 10 min.
  - Use a separate 5-mL tube for each genotype.
  - Use 4-mL sterilizing solution per tube.
  - Mix by inversion several times, tapping to release bubbles.
- Sterilized grains are rinsed three times with sterile water.
  - Again, mix by inversion and tap to release bubbles.
- Embryos are dissected from the grains under aseptic conditions and placed on medium.
  - Place the embryo with the embryo proper/coleoptile facing up and scutellum side down on the medium.
- Wrap plates twice around with Parafilm®.
- Plates with embryos are kept in the dark at room temperature for 24 h.
- Transfer to a tissue culture chamber at constant 24 °C with a 16-h photoperiod and lighting at 40 μmol/m^2/s.
- If embryos are not showing signs of germinating after approximately 4–5 days, aseptically tap them down. Some embryos do better when pressed into the medium. Some embryos flip over on the medium. This happened more frequently when embryos were directly vernalized on plates. Flipped embryos can be aseptically turned the other way up to encourage germination.
- After ~2 weeks (compared to 1 week for spring wheat), embryos are ready to be transplanted to soil. Plant donors into 2.5-cm propagation trays and let grow to two- to three-leaf stage in a growth chamber with accelerated conditions (24 h light, with a temperature regime of 16 h/8 h 22 °C/18 °C).

Growth of Donor Plants

- Sample tissue for genotyping at the two- to three-leaf stage and place trays into a vernalization chamber for 5 weeks. If, for some reason, sampling needs to be repeated while in vernalization, keep the plants chilled (as in a cold room) while sampling to avoid breaking vernalization.
- Upon receiving genotyping results, discard plants that are not positive for the desired genotype to save space.
- In contrast to the recurrent plants, after breaking vernalization and transferring to a growth chamber with accelerated growth conditions, leave the donors in 2.5-cm cells to add stress.
- Water minimally, just sufficient to avoid wilting.
- If few donors are identified, some of the positive plants can be transplanted to larger pots to encourage tillers. If transplanting is required, first place the plants

into the warmer growth chamber (24 h light, with a temperature regime of 16 h/8 h 22 °C/18 °C) for 1–2 weeks to allow acclimatization to new temperature and lighting to avoid stress.

Crossing

- Grow plants until they form heads and approach anthesis. The timing varies by line, ranging from 3 to 5 weeks between the end of vernalization and the beginning of crossing. Cross appropriately.
- Make sure that the recipient plants are emasculated prior to pollen maturation to avoid selfing. Check frequently for stamen development, especially until familiar with a donor line. The first pollen is often mature in the boot. Therefore, the spike may need to be freed from the boot for access; open boot, let dry for a day and then emasculate. Cover with a glassine grain bag to prevent inadvertent crossing.
- Crossing is successful with 2–5 days between emasculation and pollination. Recover with the glassine bags.
- The availability of pollen for crossing could change over the course of a few hours. Check often.
- Some emasculated spikes were pollinated on multiple days (from the same donor) to ensure coverage and avoid losing a line.
- After crossing, the resulting embryos can be rescued after 14 days, as described in section "Embryo Rescue of Donors" above.

8.1.9 Conclusion

The impact of accelerated breeding has been elegantly calculated in detail in terms of cost savings or increased benefits in a number of studies, covering both the DH approach for wheat (Barkley and Chumley 2012) and accelerated breeding in general (Lenaerts et al. 2018). This impact can be expressed in terms of percentages (Lenaerts et al. 2018) or in absolute dollar values, with variability depending upon the location (Brennan 1989). In all cases, the conclusions suggest that even a reduction in 1 year in terms of cultivar release is worthwhile. Hickey et al. (2019) emphasizes that speed breeding, combined with genomic technologies, will facilitate crop improvements required to feed the expected world population of ten billion. Lenaerts et al. (2019) goes so far as to suggest that a failure to adopt all available techniques to accelerate breeding would have a detrimental impact on food security and would not make economic sense.

In the present study, we suggest that accelerated breeding and DH approaches could reduce the time to release new winter wheat cultivars by 2 and 3 years, respectively. In addition, the DH methodology through isolated microspore culture has proven more efficient than the previously used maize pollination method. Protocols have been validated using diverse genotypes; differences will occur but, with slight

modifications, efficient production of DH plantlets for breeding programs will be achieved.

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Chapter 9
Genomics, Biotechnology and Plant Breeding for the Improvement of Rice Production

Kshirod K. Jena and Sung-Ryul Kim

9.1 Introduction

Rice, *Oryza sativa* L., belongs to the grass family Poaceae. It is the most important cereal crop, being the staple food of the majority of the population in Asian countries (Khush 2005; Kim et al. 2018), and it feeds more than half of the world's population. Demand for rice as a food source is rapidly increasing, and land areas for rice cultivation are also growing in African countries (Seck et al. 2012). The global population is also rapidly increasing, and it is estimated to reach nine billion by 2050. In contrast, the land area for rice cultivation is decreasing due to urbanization and industrialization, but the stability of crop production is affected due to water shortage and the adverse effects of climate change (Ray et al. 2013).

Utilizing traditional technologies, rice crop yield was dramatically increased. In late 1966, the International Rice Research Institute (IRRI) released the miracle rice IR8, with the introduction of the semi-dwarfing gene *sd1* (LOC_Os01g66100), which encodes an enzyme involved in the production of the phytohormone 'gibberellin' (GA), which affects plant height, thus increasing rice yield (Nagano et al. 2005). Over the past two decades, rice yield has been plateauing, but population increase has surpassed the rate of rice production in Asian countries. Grain yield in rice is a complex trait and is associated with different component traits, such as plant and panicle architecture, heading date, lodging resistance, tiller number, grain-filling ability, grain size, grain number per panicle, and resistance to different biotic and abiotic stresses (Kim et al. 2018).

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Advances in genetics, genomics and biotechnology in the late twentieth century had delivered significant progress in rice genome, and in the early twenty-first century, whole genome sequences of two subspecies of rice (subspecies *indica* and *japonica*) have been completed (Yu et al. 2002; IRGSP 2005). Utilizing the sequence information of rice genome, about 100 economically important genes have been cloned and characterized. Subsequently, rice (*O. sativa* L.) has evolved into a model cereal species with a 389-Mb genome size (IRGSP 2005). Additionally, with the use of next-generation sequencing technologies, 3K rice genomes have been completed (Wang et al. 2018). Of the hundreds of genes with known functions, several key genes are characterized as having definite functions related to rice yield (Kim et al. 2018; Li et al. 2018). However, good results of the evaluation of yield-positive alleles for use in the development of new rice varieties with higher yield potential have still been elusive.

In this article, we included modern strategies and methods for the development of new rice cultivars using functionally characterized genes with yield traits and precise breeding technology for the development of new rice varieties with higher yield potential.

9.2 Strategy

The Green Revolution rice, IR8, which was developed by IRRI in the 1960s had used classical genetics and breeding. The rice variety IR8 was semi-dwarf in stature, was highly responsive to fertilizer and had high yield potential. Later on, with the use of biotechnology approaches, the causal semi-dwarfing gene *sd1* was characterized as a phyto-hormone GA synthesis gene encoding GA20 oxidase-2 (GA20ox-2) for higher yield function (Sasaki et al. 2002). Since there is a need to further increase rice yield, it is imperative to adopt some strategies using recent genomics information of some key yield-related genes to realize a dramatic increase in rice yield:

1. Development of appropriate breeding methods to transfer three selected functional genes to increase yield potential.
2. Development and validation of allele-specific markers for genes expressing high-yield traits.
3. Introgression of functional alleles related to high yield into ideal genetic background.
4. Merger of genomics tools and classical breeding tools to develop high-yield cultivars.
5. Application of genome editing tools for the improvement of rice yield.

9.3 Methods, Characterization and Assays for Functional Gene Introgression

A diverse set of genes have been isolated and their functional profiles are well documented (Table 9.1) and their physical locations are mapped on the rice chromosomes (Fig. 9.1). The functions of these genes are directly connected with yield

Table 9.1 List of yield-related genes identified in rice

Gene	Protein	Traits[a]	Locus ID (MSU)	Donor allele	References
Gn1a/OsCKX2	Cytokinin oxidase/dehydrogenase 2	GN	Os01g10110	Habataki, ST12	Ashikari et al. (2005)
WFP/IPA1/OsSPL14[b]	OsSPL14	GN	Os08g39890	*WFP* allele (ST12), *IPA1* allele (Aikawa 1, Shaoniejing, Ri22)	Miura et al. (2010), Jiao et al. (2010)
GW8/OsSPL16	OsSPL16	GW	Os08g41940	HJX74	Wang et al. (2012)
GLW7/OsSPL13	OsSPL13	GL, GWE	Os07g32170	Most tropical japonica	Si et al. (2016)
qSW5/GW5	Unknown protein	GW	Deletion in NB	Nipponbare	Shomura et al. (2008), Weng et al. (2008)
Ghd7	CCT domain protein	GN, DH	Os07g15770	Minghui 63	Xue et al. (2008)
DEP1	Phosphatidylethanolamine-binding protein-like	GN	Os09g26999	Shennong 265	Huang et al. (2013)
GS3	Transmembrane protein	GL	Not annotated	Minghui 63	Fan et al. (2006)
GS5[b]	Serine carboxypeptidase	GW	Os05g06660	Zhonghua 11	Li et al. (2011a, b)
SCM2/APO1	F-box-containing protein	GN, CD	Os06g45460	Habataki	Ikeda et al. (2007), Ookawa et al. (2010)
SPIKE/LSCHL4/GPS/NAL1	Polar auxin transport	GN	Os04g52479	IR68522-10-2-2, Daringan, Nipponbare	Qi et al. (2008), Fujita et al. (2013), Takai et al. (2013), Zhang et al. (2014)
TGW6[b]	IAA-glucose hydrolase	GWE	Os06g41850	Kasalath	Ishimaru et al. (2013)
qGL3/OsPPKL1	Protein phosphatase with Kelch-like repeat domain	GL	Os03g44500	N411	Zhang et al. (2012)
GW2	RING-type E3 ligase	GW	Os02g14720	WY3, Oochikara	Song et al. (2007)
NOG1	Enoyl-CoA hydratase/isomerase	GN	Os01g54860	Guichao 2	Huo et al. (2017)
LP	Kelch repeat-containing F-box protein	GN	Os02g15950	Gamma ray radiation mutant	Li et al. (2011a, b)

[a]*GN* grain number per panicle, *GWE* grain weight, *GL* grain length, *GW* grain width, *DH* days to heading, *CD* culm diameter
[b]The three genes highlighted are ideal for high yield potential

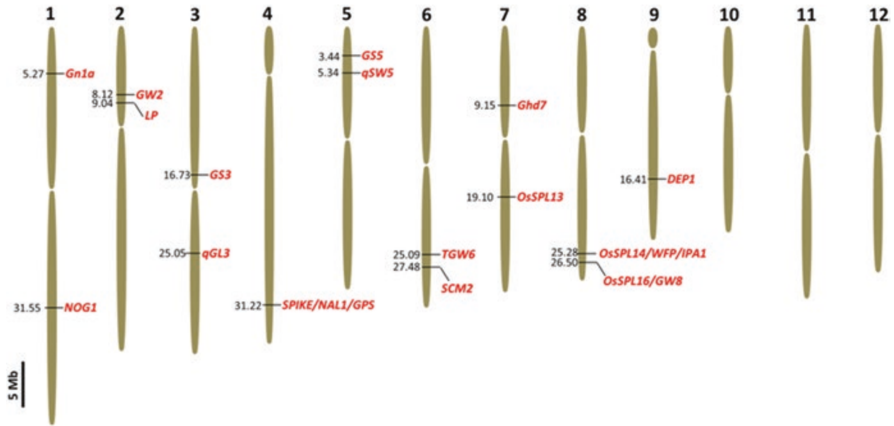

Fig. 9.1 Physical maps of identified yield-related genes that are directly involved in the regulation of grain number, grain size and grain filling in rice. The location (Mb) of each gene is calculated based on Os-Nipponbare-Reference-IRGSP-1.0 (https://rapdb.dna.affrc.go.jp/)

characteristics of rice, such as grain size, grain weight, and grain number per panicle. Sixteen key yield-enhancing genes are described for a quick understanding of their functional role in increasing rice yield.

9.3.1 Different Functional Genes for High Yield of Rice

The *Gn1a* gene-encoding cytokinin oxidase/dehydrogenase2 (OsCKX2) regulates grain number per panicle. The gene is located on chromosome 1. Reduced expression of *OsCKX2* from the *indica* high-yielding variety Habataki and the non-functional allele by 11-bp deletion on *OsCKX2* coding sequence from 5150 accession cause cytokinin accumulation in inflourescence meristems and increases grain number per panicle, resulting in enhanced grain yield (Ashikari et al. 2005).

The *IPA1*/*WFP*/*OsSPL14* gene produces ideal plant architecture/wealthy farmer's panicle/higher grain number per panicle. The gene is located on chromosome 8. The quantitative trait locus (QTL)*WFP* (*WEALTHY FARMER'S PANICLE*)/*IPA1* (*Ideal Plant Architecture 1*) encoding SQUAMOSA promoter binding protein-like (SPL) 14 negatively regulates the number of tillers in the vegetative stage and positively controls the grain number per panicle in the reproductive stage. Transcript level of *OsSPL14* in young panicles positively correlates with panicle branching and grain number per panicle. Two different yield-positive alleles (*WFP* allele from ST12 line and *IPA1* allele from the *japonica* lines Aikawa1, Shaoniejing and Ri22) exhibit a higher expression of *OsSPL14* in inflourescence with different mechanisms. In the ST12 line, *OsSPL14* transcripts were abundant because there is less DNA methylation in the *OsSPL14* promoter region (Miura et al. 2010). The *OsSPL14*-ST12 allele is regarded as an epigenetic allele that shows a heritable gene

expression difference, which is not caused by DNA sequence variations but is due to DNA methylation or chromatin status (Kakutani 2002). In contrast, the *IPA1* allele expresses OsmiR156-resistant *OsSPL14* transcripts because of nucleotide substitution (C to A) at the OsmiR156 target site located on the third exon of *OsSPL14*, resulting in a higher expression in panicles (Jiao et al. 2010). However, Aikawa1 possessing *IPA1*allele showed very low tiller number (~3 tiller per plant), although high grain number per plant is probably not much useful for yield improvement. In contrast, the *OsSPL14-WFP* allele dramatically improved grain number per panicle in all the four *indica* backgrounds tested without significant tiller number reduction, and the allele showed strong potential for rice yield improvement through the development of several high-yielding lines using the allele (Kim et al. 2018).

The *GW8* gene-encoding *OsSPL16* controls grain width (Wang et al. 2012). A higher expression of *OsSPL16* in young panicles promotes cell division, resulting in wide grains in the high-yield *indica* variety HJX74. Conversely, 10-bp deletion in the promoter region of Basmati385 causes slender grains by a reduced expression of *OsSPL16*.

GRAIN LENGTH AND WEIGHT 7 (*GLW7*) gene-encoding *OsSPL13* was identified in genome-wide association studies (GWAS) using *japonica* rice accessions. The gene is located on chromosome 7. Tandem-repeat sequence in the 5′ UTR of *OsSPL13* is involved in its expression, and a higher expression of *OsSPL13* causes large grains in tropical *japonica* rice (Si et al. 2016).

The *qSW5* (*QTL for seed width on chromosome 5*)/*GW5* gene encoding a nuclear-localized unknown protein is involved in the determination of grain width in rice. The loss-of-function allele by a 1.2-kb deletion including coding DNA sequence (CDS) of the gene increases grain width and this allele is common in *japonica* varieties (Shomura et al. 2008; Weng et al. 2008).

The *Grain number, plant height, and heading date7* (*Ghd7*) gene encoding a CCT domain protein is involved in multiple yield-related traits, including heading date, plant height and grain number per panicle (Xue et al. 2008). Nine different *Ghd7*alleles (*Ghd7-0 ~7* and *Ghd7-0a*) were identified based on predicted protein sequences from about 120 genotypes, and the *Ghd7-1* allele from the *indica* rice Minghui63 is regarded as a fully functional yield-positive allele (Xue et al. 2008; Lu et al. 2012).

The *DENSE AND ERECT PANICLE1* (*DEP1*) gene encoding a phosphatidyl ethanolamine-binding protein-like domain protein regulates grain number per panicle and panicle architecture. The gene is located on chromosome 9. The natural *dep1* mutant allele (625-bp deletion on the fifth exon) encoding C-terminal truncated DEP1 protein increased grain number per panicle, resulting in increased yield, although panicle length decreased. This allele might be derived from the Italian landrace Balilla and was distributed to many high-yielding Chinese *japonica* varieties (Huang et al. 2013).

The *GS3* gene encoding a putative trans-membrane protein functions as a negative regulator for grain size. The nucleotide substitution from C to A on the second exon caused a premature stop codon (TGA) and eventually caused the production of

C-terminal truncated GS3 proteins. This mutated non-functional *gs3* allele increased grain length (Fan et al. 2006).

The *GS5*gene encoding a putative serine carboxypeptidase regulates grain size. Different expression levels of *GS5* based on sequence variations of the promoter is associated with grain width, and based on promoter sequences, three major allele types, including wide grain, medium grain, and narrow grain alleles, were identified. Abundant transcripts of *GS5* in the hull (palea/lemma) before heading time and in the developing endosperm enhance grain width and grain weight (Li et al. 2011a, b).

The *ABERRANT PANICLE ORGANIZATION1* (*APO1*)/*STRONG CULM2* (*SCM2*) gene encoding an F-box-containing protein is involved in multiple traits, including control of rachis branching in panicles, tiller outgrowth and culm diameter. The *apo1* mutant produced smaller inflourescences with a reduced grain number per panicle (Ikeda et al. 2007). In contrast, the *APO1*-overexpressing mutant*Undulate rachi1* (*Ur1*) and *APO1*-overexpressing transgenic plants showed a dramatic increment in grain number per panicle, but tiller number per plant reduced remarkably (Murai and Iizawa 1994; Ikeda-Kawakatsu et al. 2009). The *SCM2* is a mild allele of *APO1* found in the high-yielding *indica* variety Habataki, and the allele increased grain number per panicle and lodging resistance through an increase in culm diameter without a reduction in tiller number per plant (Ookawa et al. 2010; Terao et al. 2010). This *SCM2*-Habataki allele is regarded as a useful allele of *APO1* for increasing yield and lodging resistance in a breeding programme.

The *SPIKELET NUMBER* (*SPIKE*)/*LSCHL4*/*GPS* gene is allelic to *Narrow leaf1* (*NAL1*) encoding a plant-specific protein with unknown biochemical function (Qi et al. 2008; Fujita et al. 2013; Takai et al. 2013; Zhang et al. 2014). The *NAL1*gene was originally identified through characterization of a classic rice dwarf mutant, *nal1* (Qi et al. 2008), and the other alleles were characterized by QTLs with map-based cloning by independent research groups. The *SPIKE*allele from the tropical *japonica* landrace Daringan and the *LSCHL4* allele from the temperate *japonica* variety Nipponbare increased grain number per panicle and grain yield in *indica* backgrounds (Fujita et al. 2013; Zhang et al. 2014). The protein-coding sequences of *NAL1* were identical within *japonica* varieties (Daringan, Nipponbare and Koshikari) and within *indica* varieties (IR64, 93-11 and Takanari), respectively. The *NAL1*-*japonica* allele and the *NAL1*-*indica* allele were distinguished by three single nucleotide polymorphisms (SNPs) causing amino acid change (Fujita et al. 2013; Takai et al. 2013; Zhang et al. 2014).

The *THOUSAND-GRAIN WEIGHT 6* (*TGW6*) gene encoding a novel protein with indole-3-acetic acid (IAA)-glucose hydrolase activity regulates grain weight through controlling both source ability and sink size. *TGW6* is a single-exon gene, and the loss-of-function allele (*tgw6*) caused by a 1-bp deletion in CDS, which is found in Kasalath, increased grain weight (Ishimaru et al. 2013).

The *qGL3* gene encoding a putative protein phosphatase with a Kelch-like repeat domain (OsPPKL1) is involved in the control of grain length. The rare allele of *qgl3* that leads to a long grain phenotype by one amino acid change (aspartate to glutamate transition) in a conserved motif of OsPPKL1. The near-isogenic lines (NILs)

possessing *qgl3* allele significantly increase grain yield in both inbred and hybrid rice varieties (Zhang et al. 2012).

The *GW2* gene encoding a RING-type E3 ubiquitin ligase is a negative regulator of grain size. Loss of *GW2* function by a premature stop codon caused by a 1-bp deletion on the fourth exon increased cell numbers, resulting in enhanced grain width and yield (Song et al. 2007).

The *NUMBER OF GRAINS 1* (*NOG1*) encoding an enoyl-CoA hydratase/isomerase increases grain number per panicle without a negative effect on other traits, resulting in enhanced yield. In transgenic experiments, direct transfer of *NOG1* increased grain yield by 25.8% in the *NOG1*-deficient rice cultivar, and overexpression of *NOG1* further increased grain yield by 19.5% in the *NOG1*-containing variety (Huo et al. 2017).

The *LARGER PANICLE* (*LP*) encoding a Kelch repeat containing F-box protein is involved in the regulation of grain number per panicle. Two rice recessive mutants (*lp-1* and *lp-2*) caused by gamma ray radiation in *japonica* cultivar Zhonghua11 background produced more grains and higher yield per plant (Li et al. 2011a, b).

9.3.2 Assays for the Development of Gene/Allele-Specific Markers

In this article, we targeted three potential genes (*OsSPL14*, *TGW6* and *GS5*) for transfer into an *indica* rice background. Gene/allele-specific markers are needed for tracking the target gene transfer. The development of allele-specific markers follows the procedure developed by Kim et al. (2016a). Once decided, the recurrent parents which will be improved by marker-assisted selection (MAS) with yield-enhancing genes, allele types of the target genes should be determined in the recurrent parents by application of allele-specific markers, polymerase chain reaction (PCR)/Sanger sequencing of the target genes or whole genome sequencing (WGS) methods. Because yield-positive alleles for some yield enhancing genes are widely dispersed in rice varieties (Kim et al. 2018, Jena et al. unpublished data), suggesting that target alleles for the selected genes maybe already present in the recurrents, especially in case of modern high-yielding varieties. Rare alleles, such as *OsSPL14-WFP* and *TGW6-Kasalath*, will be effective in most of the rice accessions, including *japonica* and *indica* varieties. Based on the results of allele typing of the recurrent parents, DNA marker development will be processed for absence of yield-enhancing alleles/genes in the recurrent parents. Acquisition of sequence information of the target genes from the direct donors and the recurrent parents using PCR-Sanger sequencing or WGS will be very helpful to design allele-specific markers and flanking polymorphic markers for MAS. Alternatively, obtaining target gene sequences of the rice accessions that are genetically close to the recurrent parents from public databases, such as the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) and Rice Annotation Project Database (RAP-DB) (https://rapdb.dna.affrc.go.jp/index.html), for *japonica* type reference will be helpful for

marker development. After preparation of the sequences for the target genes, DNA sequence variations between the donor and the recurrent for each gene will be screened through sequence alignment tools such as BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and NCBI blast2. Any DNA variations, including SNPs, small InDels (<20 bp) and large InDels (>25 bp), between parents can be selected for maker development, but generally functional nucleotide polymorphism (FNP), which is directly associated with phenotypes (SNPs or InDels) and large InDels located at the flanking regions, including the genes (around ±300 kb distance from the genes), are preferred for the development of allele-specific markers and for ease of marker development and clear discrimination between two alleles in agarose gel, respectively. For discriminating SNPs or very small InDels (1–5 bp) in agarose gel, cleaved amplified polymorphic sequence (CAPS) markers (Fan et al. 2009), dominant PCR markers (Kim et al. 2016a) and co-dominant tetra-primer PCR methods (Ye et al. 2001; Kim et al. 2016a) are generally used. After the development of several sets of markers, the markers should be tested with both parents and some breeding lines, including heterozygous plants, to select the clear allele discrimination and reproducible markers. Development of ideal markers is important to accurately select the target alleles and will improve the work efficiency of genotyping. Markers for three putative effective genes in *indica* backgrounds are presented in Table 9.2 as an example.

Table 9.2 The allele-specific markers for three yield-enhancing genes developed by Kim et al. (2016a)

Marker	DNA polymorphism	Primer	Primer sequence (5′ to 3′)
SPL14-04SNP	C/T SNP in promoter	SPL14-04SNP-F	TAGCCATAGCTTCTGCGTGA
		SPL14-04SNP-CR	ACCGTGCTTACCGCCtGG
		SPL14-04SNP-TR	ACCGTGCTTACCGCCtGA
GS5-indel1	4-bp InDel in promoter	GS5-indel1-F	CTAACTCCCATGGAATTACTAG
		GS5-indel1-R	GGAAAGCGAAACTGATTGACA
GS5-03SNP	T/C SNP in promoter	GS5-03SNP-OPF	ACTTTCAACTAAAGTGATATTACCTC
		GS5-03SNP-OPR	TCTATATATCCATCGTCCATGGTG
		GS5-03SNP-TF	CGCAGCCTAACTACCTAAGTAGcT
		GS5-03SNP-CR	ACATGCGTGCCAATATTCCTGTAtTG
TGW6-1d	1-bp InDel on the first exon	TGW6-1d-F	GCCAACTGATCAGACTGAG
		TGW6-1d-NR	CGTGGGGAGAGTCGATtCC
		TGW6-1d-PR	CGTGGGGAGAGTCGATtCG

9.3.3 Breeding Methods for the Precise Transfer of High-Yield Functional Genes

In our study, we used the backcross breeding method and successfully transferred several yield-enhancing genes into different indica rice variety backgrounds (Kim et al. 2018). Firstly, the recipient parent cultivar has to be selected, as well as the cultivar/line that has the target high-yield genes, to be used as the donor parent. After confirming the true hybridity of F_1 plants, first backcross progenies are to be developed through crosses of F_1 with the respective recurrent parents. Successive backcross progenies (~500 population size) have to be produced, and starting from BC_1 generation, high throughput gene/allele-specific MAS has to be applied for the selection of plants with positive marker alleles/genes of the donor parent. Progenies with homozygous alleles of the introgressed genes have to be selected for their phenotype expression for the desired traits. Some selected advanced breeding lines of BC_1F_7, BC_2F_7 and BC_1F_8 generations, along with their recurrent parent and high-yielding standard check varieties, are to be evaluated for grain yield and yield-related traits for at least three seasons in replicated trials. At the grain maturity stage, agronomic data have to be collected for net yield and yield component traits, as well as harvest index (HI), following the method described by Kim et al. (2018). Evaluate some breeding lines for their superiority on yield over the recipient parent and the standard check varieties, as illustrated in Fig. 9.2.

9.4 Trait Analysis and Product Development

9.4.1 Transfer of High-Yield Traits/Genes

We observed that most of the modern *indica* rice varieties possess high-yielding alleles of both *Gn1a* and *SCM2* genes (Kim et al. 2016a; Jena et al. unpublished data). These two genes are identified from the high-yielding *indica* variety Habataki, which probably contains IR8 (the Green Revolution rice variety) and IR24 blood based on breeding pedigree, suggesting that these two genes were continuously selected in *indica* rice-breeding programmes by breeders and were dispersed to many *indica* varieties. It means that both *Gn1a* and *SCM2* are already present in most of the *indica* varieties, and probably these genes will be effective only in most of the *japonica* varieties (Kim et al. 2018). Similarly, the *Ghd7-1* allele, *GW8* allele and *GS3* genes are common in *indica* rice varieties. On the other hand, the *OsSPL14-WFP* allele and *TGW6*-Kasalath allele are rare in rice genotypes, suggesting that these two alleles will be effective on both *indica* and *japonica* rice backgrounds. The *GS5*-wide grain alleles will be effective in *indica* rice variety backgrounds. The genes *SPIKE*, *qSW5*, *GLW7/OsSPL13* originated from the tropical or temperate *japonica* rice, and these will be effective in *indica* rice. However, the grains of *qSW5* and *GLW7* can be changed into wide grains. Yield-positive allele of *NOG1* is a bit common in *indica* rice (57.1%), but

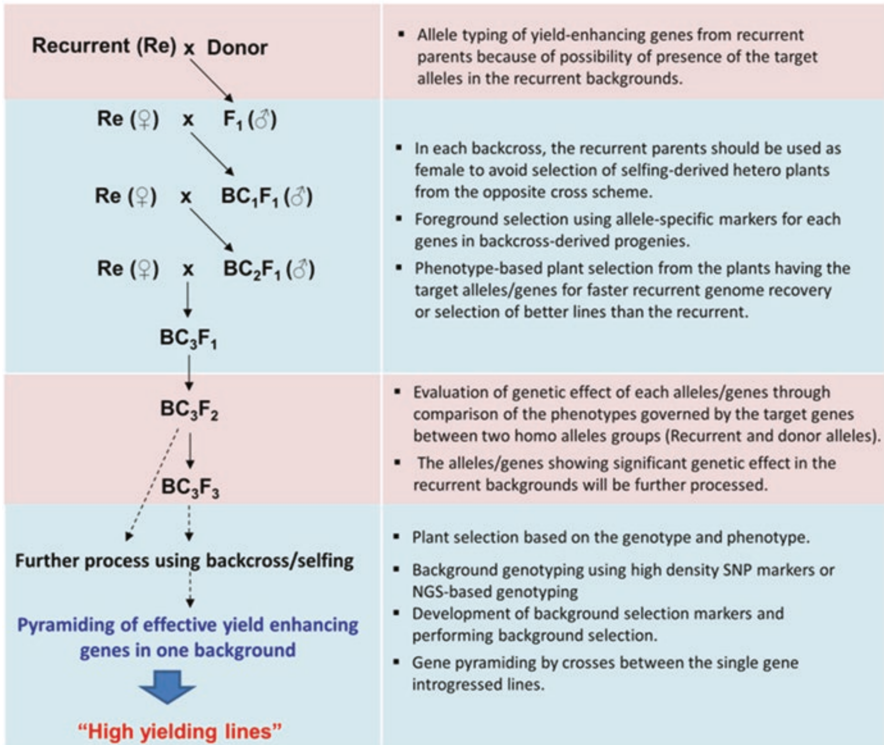

Fig. 9.2 Breeding schemes for ideal super yielding lines possessing yield-increasing genes

it is somewhat rare in *japonica* rice (17.6%) (Huo et al. 2017). Hence, the *NOG1* gene will be effective in most of the *japonica* rice cultivar backgrounds, as well as in some *indica* cultivar backgrounds. The donors of *LP* and *qGL3* are gamma ray radiation mutants developed by Chinese scientists (Li et al. 2011a, b; Zhang et al. 2012). Although these alleles are rare, accessibility of the donor gene sources will be limited. However, these mutant lines can be created using CRISPR-Cas9 tools. We have given here high-yield traits expressed by three different genes (*OsSPL14*, *TGW6* and *GS5*). These traits are expressed differently in different genotype backgrounds. A scheme showing the transfer of the three high-yield genes using high throughput allele-specific marker technology and breeding selection is provided in Fig. 9.2.

9.4.2 Foreground Selection for Presence or Absence of High-Yield Genes

For a successful and rapid transfer of high-yielding genes into *indica* rice backgrounds, it is imperative to conduct a foreground analysis of the BC_1, BC_2 and $BC_3F_{3–4}$ progenies (Jena and Mackill 2008; Kim et al. 2018). A high throughput foreground analysis of the

target genes must be conducted at the young seedling stages (at least before flowering) to select plants for further cross. For these high throughput genotyping, a simple DNA preparation method (Kim et al. 2016b) without phenol/chloroform extraction and isopropanol precipitation is very helpful. The gene/allele positive progenies are to be selected and advanced to vegetative and reproductive growth phases for phenotype characterization. The progenies with high positive correlation are to be backcrossed again to recover the homozygosity of the recurrent parent (RP) genome. It is suggested to continue three backcrosses, followed by selfing of the foreground selected progenies for evaluation of the genetic effect of the target genes using the segregating progenies (i.e. comparison of the target phenotype governed by the target gene between the donor and recurrent genotypes), which have almost the same genotype background except for the target gene. Further, these backcrosses increase RP genome recovery in the breeding lines. After evaluation of the genetic effects of the target genes, only the effective genes can be further processed. Phenotype-based plant selection in each generation from the plants that have the target alleles/genes will promote faster RP genome recovery or the selection of better lines than those of the recurrent parents with the target genes (Fig. 9.2). We have designed gene/allele-specific primers for the target yield-enhancing genes for an efficient detection of the genes/alleles in early-generation progenies from a cross between recipient and donor genotypes.

9.4.3 Background Selection and Superior Genotype Selection

Background analysis of the advanced generation progenies expressing high-yielding phenotype and having the target genes/alleles has to be carried out using suitable DNA marker technology (Jena and Mackill 2008). Background genotyping has to be conducted by using high-density SNP markers such as the Infinium 6K SNP chip (Thomson et al. 2017) or through next-generation sequencing (NGS) technology-based genotyping. Based on the background genotyping results of individual lines, background selection (BS) markers need to be developed to segregate the residual donor segments in each line through backcrossing with application of BS markers (negative selection). Genetically superior advanced backcross progenies must be characterized with the highest rate of RP genome recovery, along with the presence of the target high-yield gene at the right chromosomal locus. A quantitative estimation of the superior genotypes with their yield performance has to be selected and recommended for further yield testing.

9.4.4 Selection and Development of Ideal Breeding Lines

The application of genomics and biotechnology tools helps in developing new rice cultivars with the ability to increase yield. The selected genes we included in this article are valuable for enhancing the genetic yield potential of rice. In our study, we

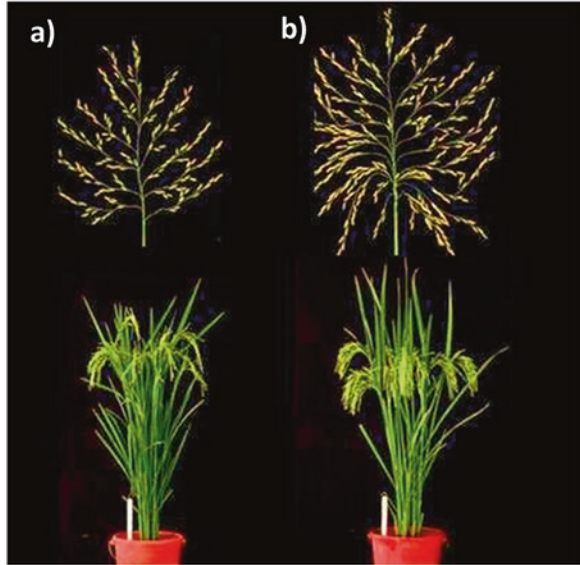

Fig. 9.3 Panicle and plant phenotypes of the recurrent parent and the improved line by introgression of *OsSPL14-WFP* allele. (**a**) One of popular indica varieties in India, MTU1010. (**b**) The improved line possessing *OsSPL14-WFP* allele in MTU1010 background

used the *OsSpl14* gene in the genetic background of the varieties PR7951, CT5803 and CT5805 and produced new high-yielding breeding lines with a dramatic increase in yield: 9.66, 11.53 and 12.89 tons per hectare, respectively. The yield was respectively 140.1%, 166.1% and 183% higher than those of their respective recipient varieties (Kim et al. 2018). Hence, the selected progenies with high-yielding attributes would bring in the production of new indica rice breeding lines with higher yield potential. Here we presented one high-yielding line possessing an *OsSPL14-WFP* allele in a popular Indian variety, MTU1010, using the above strategy, which gives a higher number of panicle branching and thus increases the number of grains (Fig. 9.3).

9.4.5 Application of Genome-Editing Tools for Rice Yield Improvement

Since the emergence of CRISPR/Cas9-mediated genome engineering in 2013 (Doudna and Charpentier 2014), genome editing (GE) of living organisms, including rice, has been achieved more easily with higher editing efficiency, compared to previous genome-editing tools such as zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs). These GE tools were applied in rice for the improvement of many important traits (Mishra et al. 2018), including biotic stress resistance, abiotic stress resistance and nutritional improvement, and also for the acquisition of novel traits, such as herbicide resistance (Li et al. 2016; Sun et al. 2016). For yield enhancement, GE tools were used for the development of

superior alleles of *Gn1a* and *DEP1* genes (Huang et al. 2018); rapid improvement of grain weight by mutation of grain weight genes, including *GW2*, *GW5* and *TGW6* genes (Xu et al. 2016); and improvement of rice productivity by mutation of the subfamily of abscisic acid receptor genes (Miao et al. 2018). As shown in the above researches, the favourite rice backgrounds can be directly improved by editing the target genes without cross with the donor parents, if transformation efficiency of the background variety is good. Also, background rice varieties can directly acquire the target alleles of yield-enhancing genes through GE tool-mediated allele replacement, although its efficiency is low at present. In the final products of MAS breeding, unexpected phenotypes are frequently observed because of the presence of flanking regions near the target gene, called 'linkage drag'. But 'linkage drag' can probably be eliminated by targeted artificial recombination induced by GE tools from heterozygous plants derived from a cross between the donor and recurrent. However, the breeding products induced by these GE tools will not be different from natural variations because the GE machinery will not be present in the final breeding products selected with high yield potential after GE. Although social regulatory issues still remain, these GE tools have strong potential for the improvement of rice productions.

9.5 Summary and Conclusions

Rice production has not increased significantly for the past three decades despite the rapid increase in the global population rate. It is expected that the global population will be about nine billion by 2050. Advances made in the field of genomics and biotechnology can help achieve a further increase in *indica* rice production through the transfer of yield-enhancing functional genes. We demonstrated here the achievements we had by transferring the yield functional gene *OsSPL14* and developing high-yielding breeding lines with superior yield potential, ranging from 28.4% to 83.5%, in the recipient varieties. Of the 16 potential yield functional genes, we focused on three genes (*OsSPL14*, *TGW6* and *GS5*) for the transfer into different *indica* rice variety backgrounds. These genes should be transferred to low-yielding *indica* rice varieties by using the strategies and methods discussed above, along with marker analysis assays and marker-assisted selection of high-yielding breeding lines. The best performing breeding lines may have some undesirable traits inherited from their donor parents, and such type of linkage drag can be corrected with the help of various emerging genome-editing technologies. New breeding lines with yield functional genes and high yield potential will help increase the production of rice to feed the increasing population of the world.

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Chapter 10
High-Frequency Androgenic Green Plant Regeneration in *Indica* Rice for Accelerated Breeding

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10.1 Introduction

The increasing growth of the world population and the gradual deterioration of the environment have become a major challenge for food security around the world, especially in rice-growing countries. Increasing rice yield has become the most important goal of rice production with less land and limited resources. Hence, there is a sustained need to focus on the development of high-yielding rice varieties with tolerance to biotic and abiotic stresses (Hasan et al. 2015). Though rice-breeding efforts over the past six decades have contributed tremendously to the genetic improvement of rice in terms of yield and quality, traditional approaches suffer from several limitations in the increase of crop yield and productivity indefinitely. Alternatively, conventional rice breeding is a slow process, which typically requires 8–10 years from initiation to varietal release, which also mostly depends on environmental conditions. Innovative biotechnologies based on the use of double haploids enable developing new varieties that are considerably faster compared to conventional plant-breeding approaches.

The doubled haploid (DH) approach not only helps in accelerating conventional plant-breeding programmes in developing early release of cultivars with superior and desirable traits, but it also has greater utility in other research aspects of plant breeding, genetics and genetic engineering. DHs are an important constituent of germplasm as well. These also help in complementing back-cross breeding by transferring genes of interest between wild relatives, thus breaking genetic barriers. Moreover, the unique and complete homozygous nature of DHs; the less time requirement to produce a large number of DHs; the absence of heterozygosity; the efficiency of the DH approach over conventional systems; and the absence of

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gametoclonal variation in DHs make DHs a very valuable material for important genetic and molecular studies. Thus, DHs are extensively used for genetic studies, like in studying the inheritance of quantitative traits, quantitative trait loci (QTL) mapping, genomics, gene identification, whole genome mapping and the production of stable transgenic plants.

Though doubled haploid technology shows several advantages over the traditional method, it requires an efficient androgenic protocol for the production of DHs in *indica* rice, which could show the notable significance similar to *japonica* subspecies. However, *indica* rice genotypes were found to be recalcitrant to tissue culture, which has been taken as a challenge for rice researchers in achieving success in developing an efficient androgenic protocol for the generation of a considerable amount of DHs.

A successful androgenic protocol envisions an efficient and fast green shoot regeneration in the production of a desirable number of DHs. It is often regulated by a conglomerate of essential factors, which play their role at every step of the anther culture. This chapter focuses on the various factors associated with androgenesis in *indica* rice with a special emphasis on green shoot regeneration, which would facilitate the achievement of DHs for accelerating the breeding programme in *indica* rice. The first step of androgenesis involves callus induction, the success of which depends upon a number of factors, such as explant genotype, medium composition, culture and growth conditions, developmental stage of microspores and pretreatment (Gioi and Tuan 2004), playing a crucial role. Phytohormones also regulate callus induction in rice androgenesis. Anthers containing microspores of suitable stages were cultured, turned light brownish and then swelled in size before calli were seen bursting the anthers and emerging asynchronously generally after 3–10 weeks of culture. N6 medium supplemented with 2,4-D (2.0 mg/L), 6-BAP (0.5 mg/L) and 3% maltose, as a sole carbon source, showed enhanced callus induction in *indica*rice hybrids. Calli bearing an off-white color upon reaching 2–4 mm in size are subjected to organ induction. These calli grow into a hard compact form before green spots start appearing on their surface. Callus induced on other forms of auxins, such as dicamba and phenylacetic acid (PAA), were proven to be detrimental in callus induction frequency, even for responsive genotypes, by ten times (Lentini et al. 1995).

10.2 Green Plant Regeneration

Out of two androgenic methods, namely one-step and two–step, that are adopted for the production of DHs (Fig. 10.1), usually two-step method is preferred in the production of DHs, either in *japonica* or *indica* rice (Silva 2010). In a two-step pathway, callus response from anther/microspore and green shoot regeneration are two important processes that should be worked out in parallel. Though there is a significant frequency of callusing response observed in *indica* rice, there is a limitation in achieving green shoot regeneration. *Indica* cultivars not only exhibit limited green

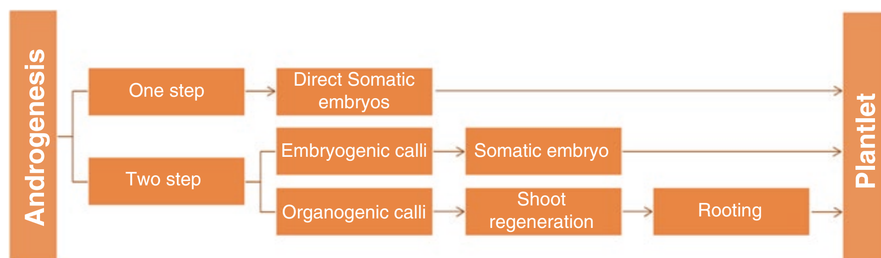

Fig. 10.1 Types of androgenesis

plant regeneration potential but also produce shoots devoid of chlorophyll that eventually die, which is known as albinos; this is the most frustrating problem, particularly in *indica* rice in the whole world. Therefore, green shoot regeneration requires more attention during androgenesis.

Green plant regeneration is considered as the baseline for achieving effective androgenesis. Often the success of androgenesis is rated based on the number of green plants regenerated per anther/microspore cultured. Achieving green plant regeneration requires a conglomeration of various factors, and the source of explants, along with callus induction media and the texture, colour and size of calli, plays a significant role in developing an efficient androgenesis method. Other factors, such as physical and chemical factors, also show significance in the increase of green shoot regeneration, either independently or in association with other factors (Fig. 10.2).

10.2.1 Source of Explants

10.2.1.1 Genotype

Success was achieved in the androgenesis of *japonica* rice, but *indica* rice was found recalcitrant to this technique (Yan et al. 1996). Moreover, the variation in androgenic response was observed between genotypes of the same ecotype (Silva and Ratnayake 2009). There was a speculation of inheritance of this androgenic trait that has been in picture for several years. Experimental evidence suggested that such trait can be inherited, which is quantitative in nature as well. Comparative studies made by Guiderdoni et al. (1992) using *indica*, *japonica* and *japonica* × *indica* hybrids clearly showed the inheritance of androgenic ability of hybrids, which are better in *japonica* than in *indica*. Conversely, the response of androgenesis was found promising in the aromatic *indica* genotypes (Suriyan et al. 2009); the performance of anther culture in aromatic genotypes was found at par with *japonica* genotypes.

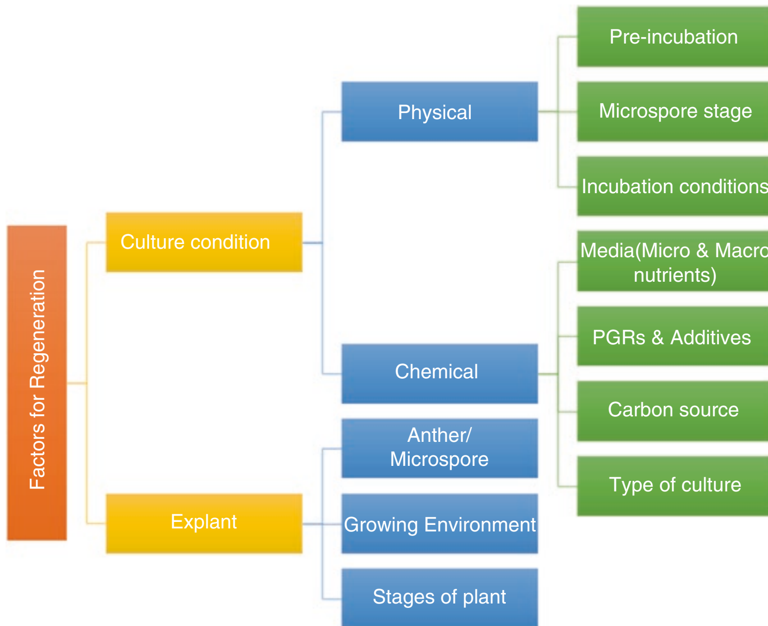

Fig. 10.2 Factors affecting green shoot regeneration

10.2.1.2 Growing Environment

Physiological growth conditions of plants control the actual number of fertile tillers that can bear healthy anthers. Microspore androgenic competence is reliant on the nutritional profile of the explant, physiological age and endogenous growth regulators. Seasonal variation in androgenic response is quite common in barley and wheat (Datta 2005). Interestingly, panicles (anthers) of field-grown plants responded efficiently to anther culture compared to the anthers collected from potted plants grown in the controlled conditions of green house (Veeraraghavan 2007). Moreover, temperature and photoperiod during the booting stage also govern the make-up of anther/microspore, which indirectly affects androgenic response. Commonly, anthers from primary tillers or early tillers are considered best for anther culture (Mayakaduwa and Silva 2018). Other factors, like pest and disease control, should be given equal priority; pesticides/herbicides have a detrimental effect on in vitro androgenic response. Guzman et al. (2000) reported an increased callusing and regeneration (70%) using anthers from ratooned plants of Taipei309 as compared to normal ones. The anthers from ratooned plants were initially cultured in an N6 liquid medium (ABA added) for 5 days, and later using an N6 liquid medium without ABA resulted in increase in callus induction. Culturing these calli onto MS medium supplemented with benzylaminopurine (BAP) (2 mg/L), α-naphthalene acetic acid (NAA) (1 mg/L) and kinetin (2 mg/L) resulted in green shoot induction for ratooned plants.

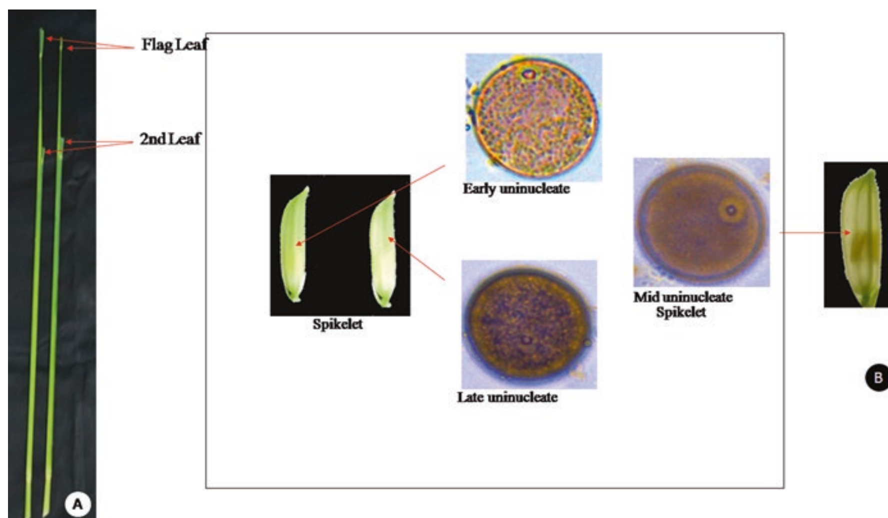

Fig. 10.3 Spikelet with different stages of microspore. (**a**) Boot leaf and (**b**) spikelet depicting anther position co-relating its microspore stage

10.2.1.3 Stages of Microspore

Though plant physiology plays an important role in the success of rice anther culture, the microspore stage is also considered as a vital factor for androgenic response. Microspores of different species respond asymmetrically to callus induction. The exact stage of microspore in which it can change its pre-programmed gametophytic stage to re-programmed embryogenic stage is quite narrow. Since the appropriate stage of microspores for anther culture is between late uni-nucleate and early bi-nucleate (Afza et al. 2000), the appropriate microspore stage for an effective androgenic response in *indica* rice hybrid (CRHR32) was reported to be early to mid uni-nucleate (Rout et al. 2016). Although microspores within an anther should be of similar age, there is difference in developmental stage, the reason being only a few microspores respond to callus formation. Since the cytological confirmation of the proper microspore stage is time consuming, the physiological indicator should be developed by correlating the microspore stage and anther position in the spikelet (Fig. 10.3). Often the distance between the collar of the flag leaf and the ligule of the penultimate leaf of the tiller serves as a reliable guide to anther maturity (Nurhasanah and Rusdiansyah 2015).

10.3 Physical Factor

10.3.1 Pre-incubation

Almost each plant species requires physical or chemical pre-treatment/stress before initiating anther culture. It is quite an essential factor for triggering the transformation of a microspore from a gametophytic pathway to a sporophytic pathway. A variety of microspore pre-treatment/stresses have been tested and found to be enhancing pollen embryogenesis, although the type, duration and time of application of these pre-treatments vary from genotype to genotype. The most commonly used anther or microspore pre-treatment conditions are temperature (cold or heat shock), sucrose and nitrogen starvation, centrifugation, as well as the use of microtubule disruptive agents such as colchicine. Lesser known stress treatments include irradiation, use of high humidity, anaerobic treatment, electro-stimulation, high pH medium, ethanol and heavy metal treatment (Mayakaduwa and Silva 2018). These pre-treatments may be classified into physical or chemical.

10.3.1.1 Temperature Pre-treatment

Cold pre-treatment is reported to stop the gametophytic development of microspores and guide the continuous division of the microspores into formation of callus/embryo, i.e. a sporophyte, during culture (Touraev et al. 1996). Effects of cold pre-treatment on callus induction include delay of anther wall senescence, increase of symmetric division of pollen grains and release of substances necessary for androgenesis, mainly amino acids and shock-thermic proteins (Kiviharju and Pehu 1998). Sometimes a shift from gametophytic to sporophytic mode of development may cause instability and the loss of chlorophyll. Cold pre-treatment of spike at 8–10 °C for 2–8 days is the most effective period for anther culture in rice genotypes (Herath et al. 2009). Moreover, pre-treatment at 10 °C for 2–8 days was found to be the most suitable condition for callusing and green plant regeneration in *indica* rice hybrid CRHR32 (Rout et al. 2016). Similarly, cold temperature treatment of spike at 10 °C for 7–8 days has a positive effect on elite *indica* rice hybrids producing green plants (Naik et al. 2017). In contrast, high temperature (30–35 °C) pre-treatment disrupts the normal integrated development of somatic anther tissue and subsequently synchronizes the physiological states of the two tissues, thereby stimulating the induction process (Dunwell et al. 1983). Though high-temperature pre-treatment is equally important, like cold, for the development of an efficient androgenic method for cereals, there are no such report available for rice.

10.3.1.2 Nutrient Starvation

Nutrient starvation, particularly of nitrogen and sugar, has been effective in enhancing in vitro anther response in some plant species. Nitrogen starvation may be applied to the anther donor plant or the excised anthers and microspores. During

starvation, cytoplasmic and nuclear changes have been observed in the microspores, including de-differentiation of plastids, changes in chromatin and nuclear structure, changes in the level of RNA synthesis and protein kinase activity, and activation of small heat-shock protein genes (Shariatpanahi et al. 2006). The mother plant can be nitrogen stressed by restricting the application of nitrogen fertilizer to the plant. Excised anthers can be starved of nitrogen by withdrawing or limiting the inorganic and organic nitrogen sources in the initial culture media. In several species, isolated microspores have shown a better embryogenic response with nitrogen starvation in the media. The culture of excised anthers under starvation and heat-shock conditions induced the formation of embryogenic microspores (Touraev et al. 1996).

10.3.2 Incubation Condition

The commonly maintained tissue culture incubation temperature of 25 ± 2 °C, the light/dark conditions, the density of anthers or microspores in a culture vessel and other such post-culture environmental conditions have a substantial effect on the success of anther culture, depending on the plant species or genotype.

10.3.3 Light and Photoperiodism

A photoperiodism nature and light duration during incubation go hand in hand. An incubation temperature of 25 ± 2 °C with 42 μmol/m^2/s illumination (cool, white fluorescent lamps) under a 16-h photoperiod is found conducive for green shoot regeneration (Rout et al. 2016). Zongxiu et al. (1993) studied the effect of thermo-photoperiod of donor plants to anther culture. Their study showed that when the plants were grown under 29.7 °C, 25.7 °C and 23.5 °C for 14.75 h, 14.00 h and 13.25 h (photoperiod), respectively, the callus induction rate was highest in anthers from the plants grown under 25.7 °C with 14-h photoperiod, while a similar temperature incubation with 13.25-h photoperiod resulted in higher green plant regeneration. On the other hand, increased temperature and photoperiod greatly reduced the callus regeneration frequency. Though the incubation was carried out at 25–29 °C during the anther culture, it may affect the callus induction and green plant regeneration. Trejo-Tapia et al. (2002) subjected callus to 8 days of dark condition and later incubated it under 40 μmol/m^2/s illumination for different media combinations, yielding a highest of fourfold increase in green shoot regeneration. In Thai aromatic rice variety, the high intensity of 70 μmol/m^2/s illumination for an initial period of 6 weeks induced green shoot regeneration in anther-derived calli (Cha-um et al. 2009).

10.3.4 Temperature and Humidity

After the initial cold pre-treatment (10 °C), culture initiation and maintenance are carried out at standard room temperature, i.e. 25 ± 2 °C. Different approaches have been made in order to study the effect of variable incubation temperatures on callus induction and shoot regeneration. Change in the incubation temperature during callus induction to 30 °C or 20 °C for a period of 14 or 10 h, instead of a continuous 25 °C, increased the callus induction rate, followed by shoot regeneration in *indica* rice (Javed et al. 2007); the rate of green plant regeneration increased from 0.00% to 0.1% and 0.5% to 4.3%.

10.4 Chemical Factors

10.4.1 Media (Micro- and Macronutrients)

Successful induction of androgenesis from cultured microspores or anthers usually depends on the macronutrients (particularly the form in which nitrogen is supplied in the medium) and micronutrients in the culture media to determine callus induction, followed by green shoot regeneration. Standard media have been developed for different species, although specific genotypes may have their individual requirements. The more widely used basal media for anther culture are N6 (Chu 1978), Nitsch and Nitsch (1969), MS (Murashige and Skoog 1962) and B5 (Gamborg et al. 1968). These media are used often in their original form but are sometimes modified by supplementing or subtracting one or more components to better suit a targeted species or genotype. When working with recalcitrant species, new media are formulated that are tailor-made to address their specific requirements.

Micronutrients in the media play a crucial role in normal plant growth and development. Deficiency symptoms arise in plants that are grown under suboptimal levels of micronutrients. As such, the tissue culture media are also formulated with the inclusion of essential micronutrients. However, in-depth studies of their influence on in vitro cell culture, particularly on androgenesis/microspore embryogenesis, are limited. This neglects their absolute requirement for many physiological and biochemical cellular processes, including the catalysis of enzymatic reactions. Though two of the micronutrients that have been investigated for their influence on microspore embryogenesis are copper and zinc in barley (Jacquard et al. 2009; Wojnarowiez et al. 2002), the effects of micronutrients are yet to be studied in rice.

10.4.1.1 Carbon Source

Carbon source is an important component in tissue culture media that compensate the energy required for the growth and proliferation of the culture. The most frequently used carbon source in tissue culture media is sucrose. Sugars also play a

crucial role for regulating osmotic pressure in the culture media, although it is secondary to its main role as energy provider. Maltose in the anther culture media hydrolyzes to glucose and degrades slowly than sucrose. On the other hand, sucrose metabolizes very rapidly into glucose and fructose. As fructose has a detrimental effect on microsporogenesis in wheat anther culture (Last and Brettell 1990; Navarro-Alvarez et al. 1994), similar pattern was observed in rice anther culture. The superiority of maltose over other sugars, such as glucose, fructose and mannitol, is well proven in rice anther culture (Bishnoi et al. 2000; Lentini et al. 1995). Exogenously supplied carbohydrates in the culture medium fulfil the osmotic requirements of the in vitro culture. Nevertheless, the type of sugar to be used is more important as an energy source rather than in osmotic regulation of the medium. Certain sugar alcohols such as mannitol and sorbitol have been used in microspore culture media purely as osmo-regulators. Both mannitol (Raina and Irfan 1998) and sorbitol (Kishor and Reddy 1986) have had beneficial effects on rice anther culture.

10.4.1.2 Nitrogen Source

An organic or inorganic form of nitrogen is supplied to the culture medium as nitrogen source. Nitrate or ammoniums are preferentially used as inorganic nitrogen, while organic nitrogen is supplied as vitamins and amino acid. Commonly, anther culture requires more than one form of nitrogen, and it is essential to balance it correctly for successful androgenesis. Chu (1978) demonstrated nitrogen in the form of ammonium ions, which were critical for androgenesis in rice and laid the foundation for the creation of an N6 medium with appropriate concentrations of $(NH_4)_2SO_4$ and KNO_3. As N6 is appraised as the best medium used for rice anther culture, specific to *japonica* types, the *indica* rice requires minimal variation to nitrogen concentration. Lower levels of $(NH_4)_2SO_4$ and increase in KNO_3 showed better anther response and green plant regeneration from anther-derived callus in *indica* rice (Raina and Zapata 1997). The organic form of nitrogen is usually found in amino acids and other protein substitutes. N6 supplemented with essential plant growth regulators (PGRs), along with vitamins, casein hydrolysate and L-proline as growth supplement, enhances green shoot regeneration (Serrat et al. 2014).

10.4.1.3 Plant Growth Regulator and Additives

Growth regulators, mainly auxins and cytokinins, are known to control the dedifferentiation processes in rice (Table 10.1). The rate of success in green shoot regeneration can be enhanced by improving the composition of the tissue culture medium, along with the manipulation of plant growth regulators (Mandal and Gupta 1995).

Table 10.1 Androgenesis in indica rice

Genotype	Cold pre-treatment	Callus induction medium	Callus induction (%)	Green shoot induction medium	Green shoot regeneration (%)	References
Oryza sativa	–	White basic medium + NAA 5 mg/L + kin 2.5 mg/L + 6% sucrose + 15% CM	–	MS + NAA 2 mg/L + kin 4 mg/L + Ads 40 mg/L + 15% CM	–	Chen and Lin (1976)
IR64 new plant type cultivars	8 °C for 8 days	N6 + 2,4 D 0.5 mg/L + NAA 2.0 mg/L	3.53	N6 + NAA 0.5 mg/L + BA 2.0 mg/L	1.12	Gioi and Tuan (2002)
Oryza sativa	6 °C for 7 days	Z2 + 2,4D 0.5 mg/L + NAA 2.5 mg/L + kin 0.5 mg/L	8.06	MS + kin 0.5 mg/L + BA 2.0 mg/L + NAA 1.0 mg/L	57–75	Shahnewaz and Bari (2004)
Amol2 and Amol3	4–8 °C for 8 days	N6 + kin 0.5 mg/L + NAA 0.5 mg/L + 2,4D 2.0 mg/L + sucrose 60 g/L	4.01	MS + NAA 1.0 mg/L + kin 4.0 mg/L + sucrose 40 g/L	3.41	Bagheri and Jelodar (2008)
Nepalese rice—Khumal-4, Pravat, Bindeswori, Hardinath-1	8 °C for 7 days	N6 + 2,4D 2.5 mg/L + kin 0.5 mg/L + $AgNO_3$ 10 mg/L + maltose 50 g/L	14.1	½ MS + NAA 1.0 mg/L + BA 2.0 mg/L + kin 0.5 mg/L + sucrose 20 g/L	2.3	Niroula and Bimb (2009)
Bg 90-2, Bg 379-2	8 °C for 14 days	N6 + 2,4 D 2.0 mg/L + kin 0.5 mg/L + sucrose 5%	0.3–7	½ MS + kin 2.0 mg/L + NAA 0.5 mg/L	2.3	Herath et al. (2009)
Thai aromatic rice	8 °C for 7–10 days	N6 + kin 2.3 µM + 2,4 D 4.5 µM + NAA 13.5 µM + maltose 87.6 mM	12.1	MS + kin 9.3 µM + NAA 5.4 µM + sucrose 87.6 mM + maltose 83.3 mM + putrescine (put) 0.5 mM + spermine 0.5 mM	78.5	Cha-Um et al. (2009)
Upland and new plant types	–	N6 + NAA 2.0 mg/L + kin 0.5 mg/L + 10^{-3}-M putrescine	–	MS + NAA 0.5 mg/L + kin 2.0 mg/L + 10^{-3}-M putrescine	29.81	Safitri et al. (2010)
Thai rice/YR8168, Thai rice/YR7633, YR4245, GH3790	7 °C for 7–10 days	N6 + 100-mg/L proline	7.03	MS + BAP 0.5 mg/L + kin 1.0 mg/L + IAA 0.25 mg/L + NAA 0.5 mg/L	16.0	Wang et al. (2015)

Boro rice hybrids	4 °C for 6–24 days	SK + AgNO 8 mg/L + casein 3hydrolysate 500 mg/L + maltose 30 mg/L	0.78–5.77	MS + BAP 2 mg/L + kin 1.0 mg/L + NAA 1.0 mg/L	8.95	Sen et al. (2011)
O. sativa, *O. brachyantha*	8 °C for 8 days	MS, N6, FJ4	7.8	Modified MS: M5, M6, SK11 and SK11M	0.68	Abbasi et al. (2011)
Habiganj Boro (Hbj B) IV and Hbj B VI	8 °C for 8–10 days	B5 + 2,4 D 1.0 mg/L + BAP 0.5 mg/L + IAA 0.5 mg/L	–	Modified B5 + NAA 1.5 mg/L + BAP 0.5 mg/L + IBA 0.5 mg/L + kin 0.75 mg/L	–	Mohiuddin et al. (2011)
Rajalaxmi and Ajay	8 ± 20 °C for 7–10 days	N6, MO-19, Sk1 + 2,4 D 2.0 mg/L + kin 0.5 mg/L + maltose 30 g/L	34.56	MS + kin 0.25 mg/L + NAA 0.25 mg/L + BAP 0.75 mg/L	20.12	Mishra et al. (2015)
Chakhao Amubi × basmati 370	10–12 °C for 10 days	N6 + 2,4 D 0.5-mg/L liquid	60	N6 + NAA 1 mg/L + BAP 2 mg/L	70	Medhabati et al. (2014)
CRHR32	10 °C for 2 days	N6 + 2,4-D 2.0 mg/L + BAP 0.5 mg/L + maltose 3%	16.35	MS + BAP 1.5 mg/L + kin 0.5 mg/L + NAA 0.5 mg/L + sucrose 30 g/L	71.66	Rout et al. (2016)
BS6444G	10 °C for 2 days	N6 + 2,4-D 2 mg/L + BAP 0.5 mg/L + maltose 3%	29.35	MS + BAP 1.5 mg/L + kin 0.5 mg/L + NAA 0.5 mg/L + sucrose 3%	68.20	Naik et al. (2017)

Auxin

Among the auxins, 2,4-diphenoxy acetic acid (2,4-D) and α-naphthalene acetic acid (NAA) are the most commonly used growth regulators for the induction of callus from rice anthers (Trejo-Tapia et al. 2002). Indole acetic acid (IAA) and NAA may induce direct androgenesis, while 2,4-D promotes rapid cell proliferation and the formation of non-embryogenic callus (Raina and Zapata 1997). Moreover, 2,4-D results in high callus induction, and 2,4-D-induced calli produce higher green plant than NAA-induced calli. While working on Mediterranean *japonica* rice, Serrat et al. (2014) achieved higher green plantlet regeneration using N6 standard salts and by lowering naphthaleneacetic acid (1 mg/L), as a sole source of auxin, as compared to cytokinin (2 mg/L). Conversely, the microspore-derived calli, of *indica* hybrid (CRHR32) when transferred to a modified MS plant regeneration medium supplemented with NAA (0.5 mg/L), kinetin (0.5 mg/L), BAP (1.5 mg/L) and sucrose (30 g/L) showed better green shoot regeneration (Rout et al. 2016). The effect of other forms of auxins (α-naphthaleneacetic acid (NAA), phenylacetic acid (PAA), picloram, dicamba) was tested individually or in association with 2,4-D for their effectiveness for in vitro androgenesis (Lentini et al. 1995). Though the 2,4-D supplemented medium was found effective in increasing callus frequency, an increase of 2,4-D concentration in the medium resulted in an abrupt proliferation of calli that do not respond to organogenesis, especially in *indica* rice (Raina and Zapata 1997); this suggests the use of low 2,4-D concentration in callus induction media.

Cytokinin

Though auxin plays a vital role in callus response, a combination of auxin and cytokinin is observed to promote green shoot regeneration in rice. A combination of 0.5 ppm IAA (indole acetic acid) and 2.0 ppm BAP facilitates the germination of androgenic embryos. Low concentration of BAP enhances the frequency of microshoots during androgenesis (Roy and Mandal 2011). Enhanced green shoot regeneration frequency for different *indica* rice hybrids, CRHR32 (Rout et al. 2016) and BS6444G (Naik et al. 2017), was observed in an MS medium supplemented with higher concentration of cytokinin, BAP (1.5 mg/L) and kinetin (0.5 mg/L) while lowering auxin (NAA, 0.5 mg/L).

Polyamines

Polyamine is a class of plant growth regulator widely distributed in various plant parts, and its abundance is realized during inflorescence. An increase in polyamine synthesis has been reported to accompany organogenesis (Aribaud et al. 1994). The role of polyamines in the induction of embryo and the regeneration of plantlets was firstly reported by Martinez et al. (2000) using gynogenic explant. Subsequently, Martinez et al. (2000) reported that polyamine treatment reduced embryo

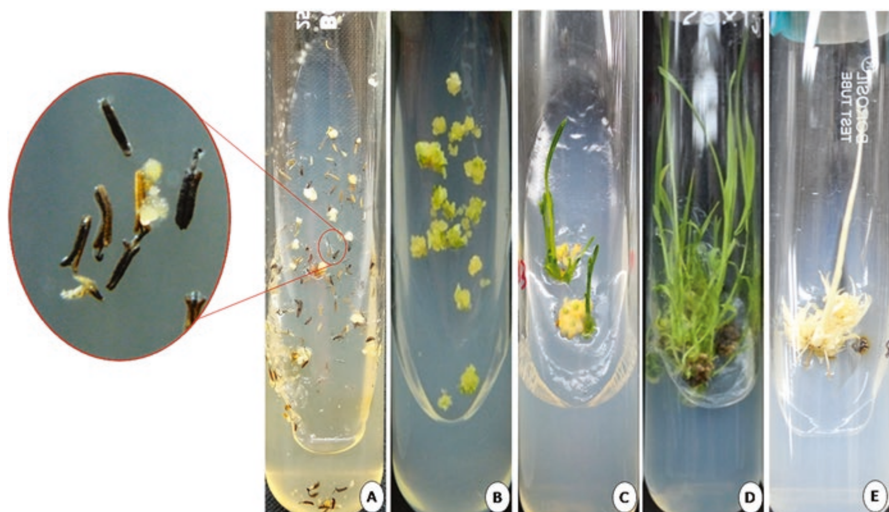

Fig. 10.4 Stages of androgenesis. (**a**) Callus induction from cultured anthers, (**b**) green spots indicating shoot emergence, (**c**) shoot elongation, (**d**) well-grown shoots, (**e**) albino shoot

regeneration time (60–90 days) compared to previously reported time (46–152 days). Though there were some genotypic differences observed in the rate of haploid production and embryo generation capacity, the highest number of callus was obtained with a treatment of 2-mM putrescine and 0.1-mM spermidine, and plantlet formation was promoted by the application of 0.1-mM spermidine post 15 days of culture. Cha-um et al. (2009) used 0.5-mM spermidine for plantlet regeneration in an anther-derived embryogenic callus, which was dependent on the plant genotype of Thai aromatic rice. Ebrahimi and Zamani (2009) also supported the findings of Martinez et al. (2000) and reported the highest number of gynogenic embryos in Iranian cultivars with an application of putrescine and spermidine. Forodi et al. (2009) also complemented the previous findings and observed that embryo production and the regeneration of plantlets increased significantly with a treatment of 0.5-mM spermidine combined with 2,4-D and BAP in the induction media. Green shoot regeneration was also found promising in putrescine added media raised calli derived from anthers of a rice hybrid (Fig. 10.4).

Other Chemicals

Silver nitrate ($AgNO_3$) also affects anther culture response in rice by inhibiting ethylene synthesis (Dewi and Purwoko 2016). Since the application of $AgNO_3$ enhanced the callus induction frequency in *indica* and *japonica* cultivars (Niroula and Bimb 2009), no significant effect in terms of callusing and shoot regeneration was observed in the rice hybrid BS6444G (Naik et al. 2017) by the addition of lower concentration of $AgNO_3$.

10.5 Types of Culture

10.5.1 Liquid and Solid Culture

The suspension culture in double haploid production was first conceptualized by Guha and Maheshwari (1964) while working on *Datura innoxia*. However, solidified media are preferred over liquid media in efficient rice androgenesis. The use of solidified media was found to enhance anther necrosis (Lentini et al. 1995), while liquid culture systems are efficient in providing microspores/calli with higher accessibility to nutrients and hormones and also dissipating toxic substances released from dying/dead anthers. However, there is a major drawback like post few days of anthers inoculation in suspension medium, anthers sink to the bottom and starts losing its viability (Raina 1997). Occasionally, ficoll is added to the suspension medium, which increases buoyancy to a level sufficient enough to keep the anthers afloat. To avoid the problems in using liquid media, solid/semi-solid media can provide sufficient support to the anthers/microspores for successful androgenesis (Gill et al. 2003).

10.6 Albinism

The *indica* cultivar exhibits limited green plant regeneration potential and often produce plants that are devoid of chlorophyll, which eventually die. The genetic make-up of donor plants is one of the major factors that affect the rate of albinism. Other factors are cold pre-treatment and media composition. Although cold pre-treatment increases the callus induction in rice, it also enhances the frequency of albino production from plantlet regeneration (Gupta and Borthakur 1987). The lack of 23S and 16S ribosomal ribonucleic acid (rRNA) in rice may cause the formation of albino plants, and these plantlets lack mature chloroplasts. Molecular analysis of albino plants showed the deletion of *rbc L* gene in plastid genome (Datta 2005). Chen (1977) achieved 92% green shoot regeneration in *japonica* rice at the early to mid uni-nucleate stage of microspore, while callus derived from late uni-nucleate yielded more albino shoots, and all regenerants were albinos when microspores at first mitosis were used. Genotype dependency was observed in both *indica* and *japonica* rice varieties. A comparative study between the crosses developed from *indica* and *japonica* genotypes showed an increase in albinism of *indica* and *indica* crosses of *japonica* (Guiderdoni et al. 1992). Recalcitrance in the *indica* genotypes could be reduced by crossing them with *japonica* genotypes. Faruque et al. (1998) attempted to increase green plant regeneration by crossing local *indica* cultivar with T-309 (*japonica* cultivar). Also, reduction in albinism was observed by minimizing the culture period of the anther-derived calli (Karim et al. 1991). The stress condition of tissue culture forced the plant cells to compete against their own plastids

with antibiotics like compounds (Torp and Andersen 2009). The role of culture medium is often subtle in albinism. Mohiuddin et al. (2011) studied the effects of callus size and the height of shoot primordial on albino plantlet regeneration in different culture media using two *indica* varieties. It was observed, when green shoots (2–3 mm) were transferred from an M10 media to the regeneration media, albino shoots recur even from the green shoots. While combining both the factors like genotypes and culture media, a rice variety, HbjB anthers cultured on M10 media without salt produced 100% albino shoots but produces 8% green shoots when cultured on KA media with salt Na_2SO_4. However, the same rice variety, HbjB produced 42% albino shoots using KA and M10 media. Rout et al. (2016) and Naik et al. (2017) reported reduced albinism and increased green shoot regeneration using an MS medium while working on *indica* rice hybrids CRHR32 (71.66%) and BS6444Gold (68.20%), respectively.

10.7 Authenticity of True DHs

A successful haploid induction requires an efficient haploid evaluation technique that could distinguish doubled haploids from unwanted heterozygous diploids. The production of homozygous lines is often accompanied by undesired heterozygous plantlets, derived from the regenerants of somatic tissues present in inoculated plant organs, such as anther wall cells, tapetum and filaments. Therefore, a reliable and fast selection technique for regenerant selection is necessary before employing tentative haploids and doubled haploids.

On a molecular level, any variations in the nuclear DNA content of cultured tissues will result in change of ploidy level, leading to phenotypic variation of the regenerants, such as triploid, tetraploid, polyploid and aneuploid. The changes include deletion, duplication and sometimes rearrangement of chromosome number in regenerated plants (D'Amato 1989). This aberration of chromosomes also influences the agronomic traits and the ploidy level of rice plants (Zhang and Chu 1984). Very often, mixoploids were observed due to the mixing of calli from different sources, like anther wall tissues and microspore calli, which are usually differentiated by examining morpho-agronomic characters; sometimes the mixploids are infertile and contain tillers of mixed type, like haploid, diploid, triploid and tetraploid (Dunwell 2010).

Different approaches are employed to assess the ploidy status in in-vitro-derived regenerants. Morpho-agronomic evaluation involves grain fertility, plant height, tiller numbers etc. The diploids or tentative DHs show normal morphological appearance and grain fertility (60–70%), while the polyploids are observed to be tall, large and bearing broad thick leaves with less than 1% spikelet fertility; on the other hand, haploids are confirmed to have diminutive stature with no spikelet fertility. Sometimes, mixploids also appear among the regenerants, which can be discriminated by observing mix tillering pattern and grain type after grain maturity

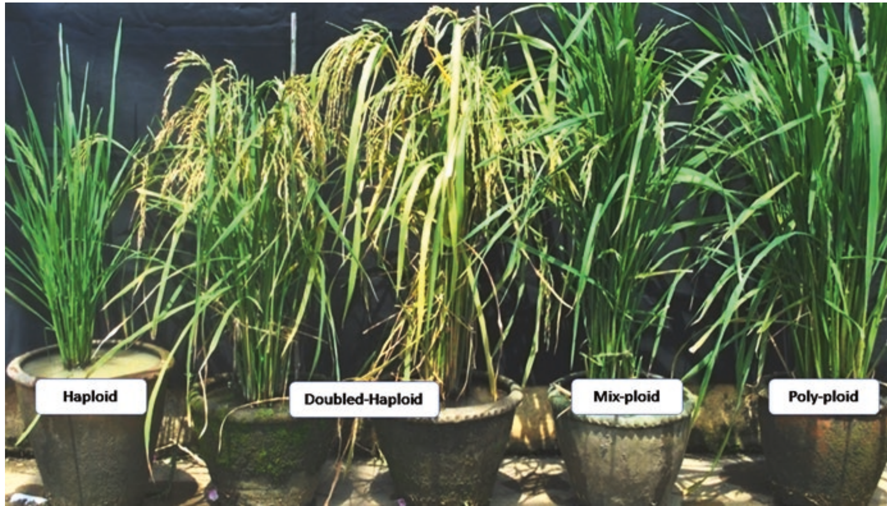

Fig. 10.5 Morpho-agronomic discrimination of developed green plants with their predicted ploidy status

(Fig. 10.5). Other techniques like cytological evaluation (Mishra et al. 2015), pollen fertility analysis (Zonneveld and Van Iren 2001) and flow cytometry/high throughput cell analysis (HTCA) (Ochatt 2008; Cousin et al. 2009; Sahoo et al. 2019) are used for differentiating ploidy status.

All the techniques cannot discriminate DHs from diploids. However, molecular markers are able to distinguish the true DHs (Rout et al. 2016; Naik et al. 2017) from the fertile diploids. Genomic molecular markers are highly conserved and heritable and often exhibit enough polymorphism to discriminate even closely related genotypes. STMSs (sequence tagged microsatellite sites) are the most preferred choice for developing genomic markers; they are abundant and well distributed throughout the rice genome. They are commonly utilized markers for genealogy (originated from microspore mother cells or embryogenesis from diploid somatic tissue), pedigree, allelic frequencies and homozygosity in plant derivatives. STMSs have been successfully utilized for the identification of homozygous, spontaneous double haploids in rice hybrids.

10.8 Artificial and Spontaneous Doubling

Haploids can be diploidized (duplication of chromosomes) in vitro to produce homozygous plants in two ways.

10.8.1 Artificial Genome Doubling

Artificial genome doubling/assisted genome doubling is a large-scale DH production technique with the application of chemical inducers. There are various chromosome-doubling chemicals, like colchicine, acenaphthene vapors, herbicide trifluralin, pronamid, amiprophos-methyl (APM) and oryzalin, among which colchicine is the most commonly used. Colchicine (tubuline polymerization inhibitor) restricts the formation of spindle fibre and forces the cell into improper division, leading to the doubling of chromosome numbers (Kleiber et al. 2012; Prasanna et al. 2012; Weber 2014). However, the major drawback to the widespread use of this chemical is its carcinogenicity and hazardous effect to the environment (Melchinger et al. 2016). Other chemicals, such as amiprophos-methyl (APM), oryzalin, pronamide and trifluralin (potential herbicides), have similar effect but is less hazardous to the environment (Wan et al. 1991; Murovec and Bohanec 2012).

10.8.2 Spontaneous Genome Doubling

Spontaneous genome doubling is a natural phenomenon in which endomitosis causes genome doubling without a division of the nucleus in androgenic (haploid) cells. Since its discovery, this attribute of haploid cells has been exploited for producing double haploid plants. The procedure involves growing androgenic calli developed from cultured microspores/anthers (haploids) in a suitable medium supplemented with growth regulators (auxin and cytokinin). This induces differentiation in the haploid calli, leading to spontaneous doubling (endomitosis), which finally results in the production of diploid homozygous cells and ultimately true homozygous plants. Spontaneous genome doubling has been reported in several cereals, including rice. The doubling rate fluctuates extremely among genotypes (Chalyk 1994; Kleiber et al. 2012). The frequency varies from 50–60% (Seguı-Simarro and Nuez 2008) to 90–99% (Naik et al. 2017) in *indica* rice. Haploid cells in general are unstable in culture with a tendency to undergo endomitosis.

10.9 Rooting and Acclimatization

Root induction is a part of organogenesis that mainly depends on various concentrations of PGRs. MS media supplemented with increased cytokinin to auxin ratio promotes root development in regenerants. Usually agar concentration is kept a notch higher than in the regeneration medium so as to mimic soil density. The MS

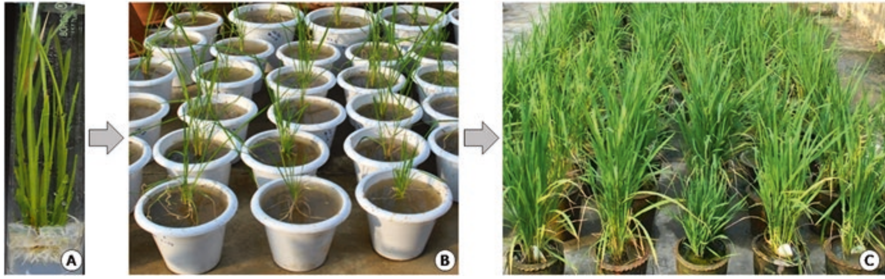

Fig. 10.6 Rooting in micro-shoots and acclimatization of anther-derived green plants. (**a**) Well-formed roots, (**b**) acclimatization, (**c**) in-vitro-generated green plants grown in net house showing variability

medium is supplemented with NAA (2.0 mg/L), Kn (0.5 mg/L) and 5% sucrose, along with 0.8% agar for rooting, i.e. 0.2% more than that in the regeneration medium. Incubation conditions are kept similar to regeneration conditions, i.e. 25–27 °C, in 16 h light with an intensity of 2000–3000 lux and 8 h in the dark under 60–65% relative humidity. The well-formed rooted plantlets are subjected to acclimatization, which allows the plantlets to cope with field conditions. The roots of the plantlets are kept in Hoagland solution overnight (12 h) at 25 °C. These plants are later transferred to garden soil (vermicompost:soil = 1:3) with three split doses of nitrogen, phosphorous and potassium (NPK) (0.75 g:0.3 g:0.36 g) in the net house (Fig. 10.6).

10.10 Field Performance of Doubled Haploids

DH technique was used to overcome the constraints associated with *indica* rice hybrids: (1) expensive seed, depriving Indian marginal farmers to utilize the seed year after year, and (2) unpredictable environmental condition and asynchronized flowering. Standardization of DH technology in rice hybrids PHB71 and KRH2 could generate promising DHs that were released as varieties, Satyakrishna and Phalguni in 2008 and 2010, respectively. Subsequently, the development of an efficient androgenic protocol for two *indica* rice hybrids, i.e. CRHR32 (an elite long-duration *indica* rice hybrid developed at the National Rice Research Institute (NRRI), Cuttack) and BS6444G (a popular rice hybrid, Bayer Seed Pvt. Ltd.), generated a considerable amount of promising DHs (Rout et al. 2016; Naik et al. 2017), which showed at par grain yield with that of parent rice hybrids; the grain quality of the DHs was also observed to be better than the quality of rice hybrids (Fig. 10.7).

Fig. 10.7 Field evaluation. (**a**) DHs derived from CRHR32 and (**b**) DHs derived from BS6444G

10.11 Conclusion

The recalcitrant nature of *indica* rice requires the optimization of anther culture methods for *indica* rice, which is very much important to achieve the potential yield of doubled haploid technology. Though a considerable increase in green shoot regeneration was demonstrated by Rout et al. (2016) and Naik et al. (2017), there is a need to further increase the callusing potential of anthers from *indica* rice. Simultaneously, direct somatic embryogenesis from microspores needs to be focused on, as this method is considered as cost-effective among all the pathways involved in tissue culture. Finally, the mechanism of spontaneous chromosome doubling in androgenesis requires immediate attention.

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Chapter 11
Doubled Haploid Technology for Rapid and Efficient Maize Breeding

Vijay Chaikam and B. M. Prasanna

11.1 Introduction

Maize (*Zea mays* L.) is adapted to and grown across diverse agro-ecologies worldwide and has the largest production globally among all the cereal crops (Fischer et al. 2014). The crop is important for food security in some of the poorest regions of Africa, Asia, and Latin America (Prasanna 2011; Shiferaw et al. 2011). Recent data indicate that 60–70% of the areas cultivated with maize are in the developing world, mostly in low- and lower-middle income countries (FAO 2018). It has been estimated that by 2050, the demand for maize in the developing world will double, and this underscores the need to accelerate yield growth (Rosegrant et al. 2009; Prasanna 2011; Shiferaw et al. 2011). Maize yields have increased tremendously in the corn-belt region of the USA since the 1930s, and at least 50% of this increase has been attributed to the genetic improvement of maize (Cardwell 1982; Duvick 2005). Increase in the maize yields in the USA and several other countries is concomitant with the adoption of hybrid maize varieties. Demand for hybrid seed is now increasing in the developing world, leading to the development of a more robust private seed industry (Fischer et al. 2014). It is very important to quickly develop and deploy high-yielding and stress-resilient hybrids with desired end-use quality, mainly in the tropical regions, for productive, sustainable, and resilient maize-based cropping systems, especially in the face of climate change.

Hybrid breeding exploits heterosis in yield and other traits that result from crossing two genetically dissimilar inbred lines. Development of productive inbred lines is, therefore, an integral part of hybrid maize breeding programs. During the last century, the development of near-homozygous inbred lines in maize relied almost exclusively on recurrent selfing and selection to reach the desired level of

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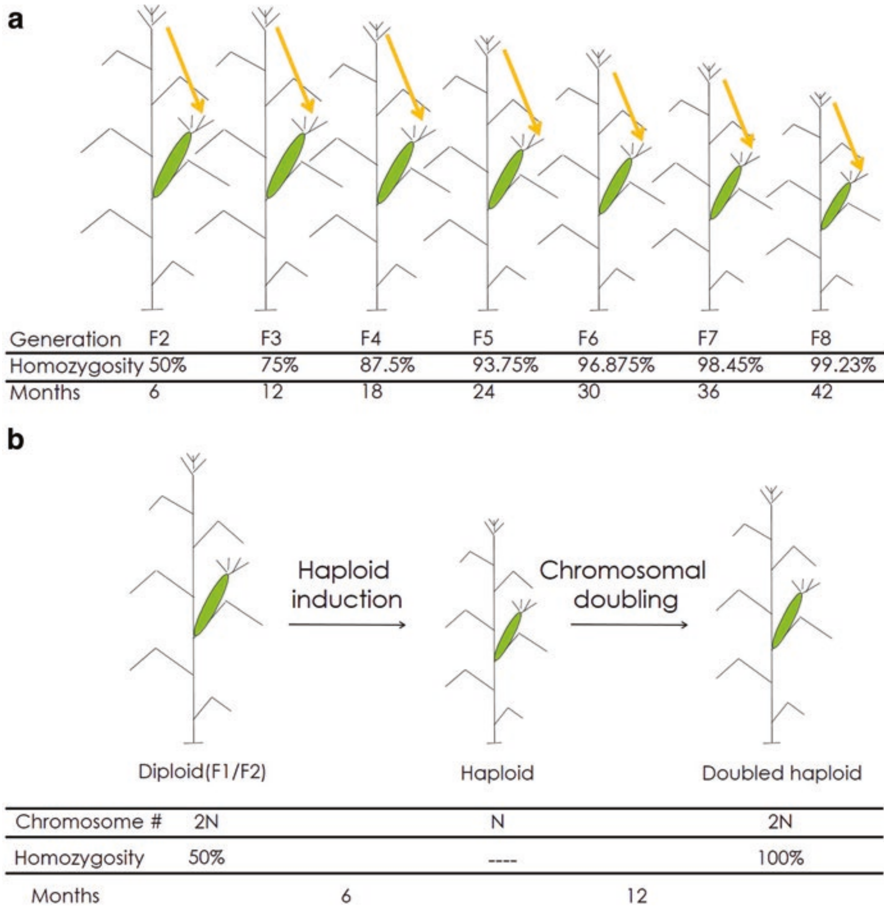

Fig. 11.1 (**a**) Illustration of conventional inbred line development based on recurrent selfing approach. Homozygosity (%) at each selfing generation and the time taken to reach each advancement stage are also depicted. (**b**) Illustration of maize DH line development. Homozygosity (%) of the source population was assumed to be 50%

homozygosity (Hallauer et al. 2010). These require six to eight generations of inbreeding after crossing to generate inbred lines suitable for extensive evaluation (Fig. 11.1a). This method has the advantage of enabling selection and further genetic recombination in the selfing generations but is very labor and time intensive, even with the use of off-season nurseries. This process often takes 11–13 years between making crosses to releasing cultivars.

Maize breeders and geneticists have been seeking ways to speed up the process of inbred line production. In the last two decades, doubled haploids (DHs) have emerged as potential alternatives for conventional inbreds as parental inbred lines for hybrid breeding and for use in maize genetics. The process of DH line production involves generating haploids with only the gametic number of chromosomes from diploid

segregating populations, followed by the doubling of the chromosomes in the haploids so that the final line contains two sets of chromosomes that are exact replicas of each other, thus making the line completely homozygous at all loci. Conceptually, this process is simple and can be completed in two generations, thereby significantly reducing the time for generating homozygous lines (Fig. 11.1b). Use of DH lines is recognized as an important means for enhancing breeding efficiency. Temperate maize breeding programs have led the way in adopting DH technology, with many commercial maize breeding programs in Europe (Schmidt 2003), North America (Seitz 2005), and China (Chen et al. 2009) using the technology. Maize breeding programs in tropical environments in the developing world have lagged in producing and using DH lines (Prasanna et al. 2010; Kebede et al. 2011). However, the situation is rapidly changing, with intensive efforts by the International Maize and Wheat Improvement Center (CIMMYT) on several fronts, including fine-tuning of the DH technology for tropical/subtropical environments and developing DH facilities in Kenya and Mexico that offer DH line production services for maize breeding programs operated by CIMMYT, the National Agricultural Research Systems (NARS), and small- and medium-enterprise (SME) seed companies, besides capacity building of the partner organizations in maize DH production and use. This review focuses on the maize DH line production process, the technical advances at each step of DH line production, and the benefits of using DH lines in maize breeding.

11.2 Procedures for the Development of Maize DH Lines

The process of DH line production typically involves the production of haploids by in vitro and in vivo methods, the identification of haploids from diploids, chromosomal doubling in identified haploids, and seed production from fertile DH plants.

11.2.1 Induction of Haploids

11.2.1.1 In Vitro Production of Haploids

Both in vitro and in vivo methods have been employed to obtain maize haploids. In vitro methods based on androgenesis involving pollen or anther culture and gynogenesis involving ovary or ovule culture have been reported in maize. However, relative to in vitro androgenesis, in vitro gynogenesis has rarely been reported, and the literature indicates a very low frequency of haploid induction in in vitro gynogenesis (Ao et al. 1982; Truong-Andre and Demarly 1984; Tang et al. 2006). Consequently, in vitro gynogenesis is not an effective method of producing maize DH lines. Following the success of in vitro androgenesis in many plant species (Forster and Thomas 2005; Dunwell 2010), extensive research has been carried out to optimize androgenesis for maize DH line production. However, obtaining

haploids and DHs through androgenesis was found to be genotype dependent and not quite reliable, with most maize germplasm being recalcitrant and the response dependent on many factors, including anther stage, anther pretreatment, technical efficiencies, etc. (Brettel et al. 1981; Genovesi and Collins 1982; Pace et al. 1987; Wan et al. 1991; Büter 1997; Barret et al. 2004; Spitkó et al. 2006). In addition, DH line production using androgenesis requires laboratory and sterile grow-room facilities, along with a skilled staff.

11.2.1.2 In Vivo Haploid Induction

In contrast to in vitro haploid induction, in vivo haploid induction has been widely utilized in maize, especially over the last three decades. Almost all DH production pipelines in commercial and public sector breeding programs are now based on in vivo haploid induction. Interest in in vivo haploid induction was initially triggered by the discovery of naturally occurring haploid plants in maize plantings during the 1920s–1960s (Chase 1969). Some of the commercial parental lines and hybrids developed in the USA and Europe during the 1950s and 1960s were based on spontaneously occurring haploids (Chase 1969). However, the low frequency (~0.1%) of spontaneous haploid induction and the inability to efficiently identify the resulting haploids and to double the chromosomes in them made it difficult to produce DH lines on a scale required by maize breeders. A major advancement in DH production technology came when certain maize genotypes were identified as having increased frequencies of haploid induction, i.e., at least tenfold higher than the natural occurrence (Coe 1959; Kermicle 1969). Genotypes that can enhance the frequency of haploids are generally referred to as “haploid inducers.” Haploid inducers are classified into two types, namely paternal and maternal inducers.

Paternal Haploid Induction

Paternal haploids are induced when the source germplasm from which haploids are desired is crossed as a male parent with the inducer female parent. Paternal haploids carry the genome of the male parent. The gene conditioning paternal haploid induction was identified as *ig1* (*indeterminate gametophyte 1*) (Kermicle 1969, 1971; Evans 2007). Paternal haploid induction is not widely used in maize breeding programs because the haploid induction frequency is low (~1–2%) (Pollacsek 1992; Kermicle 1994) and the resulting haploids inherit cytoplasm from the inducer rather than the source germplasm (Kermicle 1973). However, induction of paternal haploids can be used for the conversion of an inbred line into a cytoplasmic male sterile version (Schneerman et al. 2000; Geiger 2009; Weber 2014).

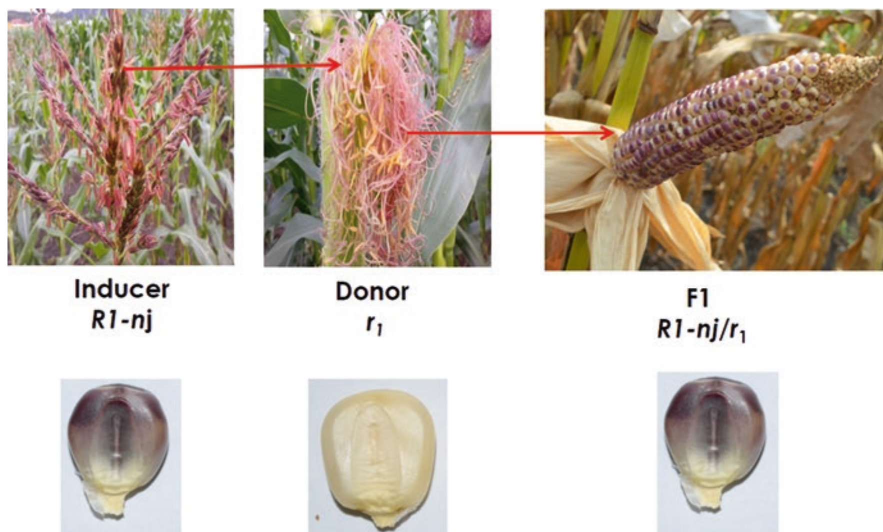

Fig. 11.2 Illustration of maternal haploid induction cross in maize

Maternal Haploid Induction

Maternal haploid induction is based on haploid inducers that are used as pollen parents and are crossed to source germplasm that is used as a female parent (Fig. 11.2). Maternal haploids thus inherit both the cytoplasm and nucleus from the same female parent, and thus maternal haploid induction is preferred by maize breeding programs. Almost all DH line production pipelines in the public and private sectors are now based on maternal haploid induction. The influence of the paternal genotype on haploid induction was first described by Chase (1949). Coe (1959) reported that stock 6 produced maternal haploids at a frequency of 1–3%, which was the highest haploid induction rate (HIR) reported up to that time. Stock 6 can be considered as the first maternal haploid inducer that was identified with HIR higher than spontaneous generation. Subsequent breeding of new inducer stocks to improve the HIR and adaptation to different environments led to the development of several new stock-6-based maternal haploid inducers with high HIR (Sarkar et al. 1972; Aman and Sarkar 1978; Zavalishina and Tyrnov 1984; Liu and Song 2000; Shatskaya 2010). Based on stock-6-derived haploid inducers, several temperate inducers with high HIR (6–15%) were developed, including UH400 (https://plant-breeding.uni-hohenheim.de/84531#jfmulticontent_c167370-2), RWS (Röber et al. 2005), MHI (Chalyk 1999), PHI (Rotarenco et al. 2010) which are being extensively used in DH production pipelines in commercial maize breeding programs.

In the tropics, haploid inducers suitable for large-scale haploid inductions were not available until recently. When temperate inducers are grown in the tropics, they

show poor vigor, susceptibility to tropical diseases, very early flowering, and poor yield, making it difficult to maintain these lines and use them for effective haploid induction (Prigge et al. 2012a). To address these problems, CIMMYT in collaboration with the University of Hohenheim developed first-generation Tropically adapted haploid inducer lines(TAILs) that have superior agronomic performance compared to temperate inducers, as well as an HIR of 6–9% (Prigge et al. 2012a; Chaikam et al. 2016). However, there was substantial scope to further improve the HIR and agronomic performance of TAILs. CIMMYT has thus developed second-generation TAILs (CIM2GTAILs) that have much superior HIR (9–14%) and agronomic characteristics compared to first-generation TAILs (https://www.cimmyt.org/second-generation-haploid-inducers-now-available/) (Chaikam et al. 2018). Using CIM2GTAILs, the number of plants of source germplasm required for haploid induction can be reduced substantially compared to the first-generation TAILs. CIM2GTAIL hybrids can also be effectively used in spatial or temporal isolation in haploid induction nurseries due to their superior plant vigor and pollen production capabilities, thereby reducing the costs associated with manual pollination.

Generally, DH production pipelines rely on inducer hybrids for haploid inductions because the hybrid inducers show superior agronomic performance and better pollen production compared to inducer inbreds. Typically, the HIR of hybrid inducers is within the range of the HIR of inducer inbred lines. At CIMMYT, a second-generation haploid inducer hybrid is currently being used for large-scale haploid inductions. Inducers are stagger planted four to five times at 5- to 7-day intervals to achieve synchrony among maize populations of diverse maturity groups.

11.2.2 Maternal Haploid Induction Associated Traits

Maternal haploid induction entails several reproductive abnormalities. In crosses involving noninducer and inducer genotypes, a higher proportion of noninducer plants are observed in the progenies than would be expected according to Mendelian inheritance, indicating segregation distortion against the inducer genotypes (Barret et al. 2008; Prigge et al. 2012b; Dong et al. 2013, 2014; Nair et al. 2017; Chaikam et al. 2018). Both gametophytic and zygotic selection have been noted as contributing to segregation distortion, and a maternal haploid inducer with a high HIR is associated with high levels of segregation distortion (Xu et al. 2013). As haploids show fitness disadvantages and are less likely to be reproductively successful, natural selection may disfavor the haploid induction trait, resulting in segregation distortion (Prigge et al. 2012b).

Occurrence of defective kernels with endosperm and embryo abortion is commonly observed when using inducer pollen for selfing and crossing compared to when using noninducer pollen (Fig. 11.3) (Prigge et al. 2012b; Xu et al. 2013; Qiu et al. 2014; Kelliher et al. 2017; Li et al. 2017; Nair et al. 2017; Chaikam et al. 2018; Tian et al. 2018). Inducers with high HIR also result in high rates of defective kernels (Li et al. 2017; Chaikam et al. 2018), and most aborted seeds carry the inducer

Fig. 11.3 Depiction of maternal-haploid-induction-associated traits, namely, kernel abortion, embryo abortion, kernels with twin embryos, and twin plants

genotype at a major locus critical for haploid induction (discussed below) (Xu et al. 2013). Consequently, similar genetic mechanisms can be assumed to be responsible for haploid induction and abortion (Xu et al. 2013). Kernel abortion might happen during fertilization or postfertilization, and it can be the result of unsuccessful double fertilization or the adjournment of zygote or endosperm development. Abortion of kernels with the inducer genotype may play a critical role in causing the segregation distortion associated with haploid induction.

A high frequency of heterofertilization where the egg cell and central cell are fertilized by sperm cells from different pollen grains is noted when using the inducer pollen (Sarkar and Coe Jr 1966; Rotarenco and Eder 2003; Liu et al. 2017; Tian et al. 2018). Heterofertilization can be the result of delayed fertilization caused by the poor competitive ability of inducer pollen (Liu et al. 2017). Heterofertilization may be the consequence of compensating for the initial failure of double fertilization by accepting a second pollen tube (Dresselhaus and Sprunck 2012; Liu et al. 2017).

Another reproductive anomaly associated with maternal haploid induction is the occurrence of twin plants at high frequencies (Fig. 11.3) (Sarkar and Coe Jr 1966; Chase 1969). Twin plants can be diploid-diploid, diploid-haploid, and haploid-haploid (Sarkar and Coe Jr 1966). The HIR of an inducer has been correlated with the rate of twin embryo seeds, and most twins are developed from the cleavage of a developing embryo (Liu et al. 2018).

11.2.3 Genetics of Maternal Haploid Induction

Maternal haploid induction is a quantitative trait controlled by a few genes (Lashermes and Beckert 1988). In noninducer × inducer crosses, several quantitative trait loci (QTL) influencing haploid induction have been identified (Barret et al.

2008; Deimling et al. 1997; Prigge et al. 2012b). Deimling et al. (1997) conducted the first QTL mapping study that identified two QTLs on chromosomes 1 and 2, which explained 17.9% of the phenotypic variance for the haploid induction trait. A segregation-distortion-based QTL mapping method employed by Barret et al. (2008) revealed a major QTL on chromosome 1. Prigge et al. (2012b), by using four mapping populations, identified two major QTLs, *qhir1* on chromosome 1 (bin 1.04) and *qhir8* on chromosome 9 (bin 9.01), which explained 66% and 20% of the genetic variance for haploid induction, respectively. This study also revealed six minor QTLs. All the QTL mapping studies mentioned above indicated that the bin 1.04 region that includes *qhir1* QTL has a major effect on haploid induction; therefore, this QTL was considered to be critical for conditioning haploid induction. Fine-mapping efforts narrowed it down to a region of 243 kb in length (Dong et al. 2013). Using 53 inducers and 1482 noninducers, Hu et al. (2016) conducted a genome-wide association study (GWAS) that led to the separation of *qhir1* into two regions, *qhir11* and *qhir12*, and *qhir11* was defined within the region fine mapped by Dong et al. (2013). However, further evaluation of the effects of *qhir11* and *qhir12* on the HIR confirmed that only *qhir11* had a significant influence on haploid induction (Nair et al. 2017). Three independent studies reported cloning the gene responsible for the haploid induction trait within the *qhir11* region and named the gene MATRILINEAL (MTL) (Kelliher et al. 2017), NOT LIKE DAD (NLD) (Gilles et al. 2017), and ZmPLA1 (Gilles et al. 2017). This gene encodes a sperm-specific phospholipase A protein. Different methods like backcrossing, TALEN technology, or CRISPR/Cas9 technology have been used to validate the effect of a mutant allele of this gene, and this revealed wide variation in the extent of haploid induction (0.5–12.5%) conditioned by the mutant allele. The variation in the HIR points to its quantitative nature and the effects of other possible genes in the genetic background on maternal haploid induction. Further studies are needed to establish the mechanism by which this specific protein triggers maternal haploid induction. Recently, the gene underlying the second most important QTL, *qhir8*, was also cloned and identified as ZmDMP gene encoding a DUF679 domain membrane protein (Zhong et al. 2019). Even though the mutant allele conditions poor HIR (0.1–0.3%) by itself, it dramatically increases the HIR conditioned by the mutant allele of the MTL/NLD/ZmPAL1 gene by five- to sixfold. These results indicate that the mutant allele of MTL/NLD/ZmPAL1 is most critical for conditioning haploid induction, and genes underlying other QTLs can enhance the effect of this allele on HIR.

Other than the haploid inducer, HIR is also noted as being influenced by the source germplasm from which the haploids are derived (Kebede et al. 2011). This phenomenon is generally referred to as haploid inducibility. Significant variation for HIR was observed across elite tropical germplasm, and higher induction rates were recorded in some single-crosses and landraces than in open-pollinated varieties (OPVs) (Prigge et al. 2011). Other studies also indicated significant variation in the maternal genotypes for haploid inducibility (Wu et al. 2014; De La Fuente et al. 2018). Two QTLs for haploid inducibility were identified in the source germplasm that explain 14.7% and 8.4% of the total genetic variance of the trait, respectively (Wu et al. 2014). Analysis of maternal parent influence on HIR using 672 elite

inbred lines at CIMMYT also revealed great genetic variation for haploid inducibility in tropical germplasm (Nair et al. 2020). A genome-wide association study for haploid inducibility in these tropical lines led to the identification of several genomic regions that affect haploid induction. A few of the tropical inbred lines have shown very positive responses to haploid induction with an HIR that is more than double the mean HIR of all of the lines. These inbred lines could be potentially used to improve the haploid inducibility of germplasm relevant to maize breeders.

11.2.4 Possible Mechanisms of Maternal Haploid Induction

The mechanism and exact sequence of events conditioning maternal haploid induction is yet to be conclusively elucidated. As early as in 1959, it was evident that the pollen of maternal haploid inducers was responsible for inducing haploids (Coe 1959). Subsequently, several studies explored whether there were any abnormalities in pollen from haploid inducers. Monospermy in inducer pollen was dismissed as a cause of haploid induction after analyzing thousands of pollen grains from inducer stock 6 because they were all normal with three nuclei (Sarkar and Coe Jr 1966). Also, the possibility of two haploid sperm cells or a single diploid cell fertilizing the central cell was ruled out because endosperm in haploid seeds was determined as triploid rather than tetraploid (Chase 1964a, b; Sarkar and Coe Jr 1966). However, certain phenotypic abnormalities such as high frequencies of two pollen tubes instead of one (Pogna and Marzetti 1977) and high proportions of pairs of morphologically different sperm nuclei (6.3%) (Bylich and Chalyk 1996) were recorded when using inducer pollen. Such differences in sperm nuclei might arise from two sperm cells developing at different speeds, leading to a state where one sperm is ready for fertilization and the other is not. Other reproduction anomalies associated with haploid induction include high numbers of microsporocytes with aneuploidy (Chalyk et al. 2003; Qiu et al. 2014; Li et al. 2017) and poor competitive ability of the inducer pollen compared to noninducer pollen in dual pollination experiments where the same silks were pollinated by inducer pollen, followed by noninducer pollen (Xu et al. 2013). Poor competitive ability of inducer pollen was attributed to delayed pollen germination.

As described above, several reproductive anomalies associated with haploid induction could be the result of defects in the pollen or sperm cells. Higher levels of endosperm/embryo abortion and heterofertilization when using inducer pollen point to single fertilization instead of normal double fertilization. Studies of embryogenesis after pollination with haploid inducer pollen using advanced microscopy identified single fertilized ovules (Swapna and Sarkar 2012; Xu et al. 2013; Tian et al. 2018) providing evidence for single fertilization. These observations indicated that defective fertilization processes occur with inducer pollen where one sperm fuses with the central cell but the other sperm cell does not fertilize the egg cell. Despite this, the egg cell is triggered to become a haploid embryo through influence from either the dividing central cell or the attenuated sperm cell.

In contradiction to the above observations, there is also proof for occurrence of normal double fertilization involving inducer pollen. The most important evidence in this regard is the observation that maternal haploids and the DHs resulting from them often contain inducer chromosome segments (Fischer 2004; Zhang et al. 2008; Li et al. 2009; Zhao et al. 2013; Qiu et al. 2014). This observation corroborates the occurrence of normal double fertilization and loss of inducer chromosomes from the developing embryo. Further evidence for double fertilization and subsequent elimination of chromosomes came from the transmission of phenotypic, physiological, or genetic markers integrated in the haploid inducers to the haploids. When using an inducer equipped with the seed anthocyanin marker and a high-oil marker, haploids with weak anthocyanin expression and high oil content were detected at low frequency, indicating that the chromosomal segments conditioning these traits were integrated into the haploids (Li et al. 2009). Similarly, when inducers transformed with cytogenetic markers like the B chromosome were used in induction crosses, B chromosomes were observed in haploids at a low frequency (Zhao et al. 2013). Further, when a sweet corn line with a shrunken endosperm was crossed with an inducer with a normal endosperm, some hybrid kernels showed a mosaic endosperm consisting of a normal endosperm and a shrunken endosperm, and this indicated the loss of inducer chromosomes (Zhang et al. 2008). Other evidence for chromosome loss during haploid induction includes the occurrence of anomalies like aneuploidy, mixoploidy, lagged chromosomes, and micronuclei in mitotic cells of ovules and developing embryo/endosperm (Wedzony et al. 2002; Chalyk et al. 2003; Zhang et al. 2008; Zhao et al. 2013; Qiu et al. 2014; Li et al. 2017). Together, many of these studies point to multiple mechanisms of maternal haploid induction.

11.2.5 Breeding for Maternal Haploid Inducers and Their Maintenance

Great progress has been made in the development of maternal haploid inducers under a range of agroclimatic conditions. However, there is still scope to improve HIR, integrate new markers for haploid identification, and improve agronomic performance of haploid inducers in specific environments. HIR can be improved by a selection of transgressive segregants that show higher HIR than that of the parents in crosses involving either two inducers (Röber et al. 2005) or an inducer and a noninducer (Chaikam et al. 2018). A pedigree method that involves a selection of individual plants based on highly heritable traits in the F_2 generation, followed by a selection among the families for haploid induction rate, is suggested as an effective method for inducer development (Prigge et al. 2012a). If improvement of adaptation for a specific environment is the main objective, backcrossing the F_1 to the adapted parent can be used without necessarily sacrificing the HIR (Prigge et al. 2012a; Chaikam et al. 2018). In both selfing and backcrossing methods, improvement of many traits relevant for the tropics has been demonstrated in inducer × noninducer

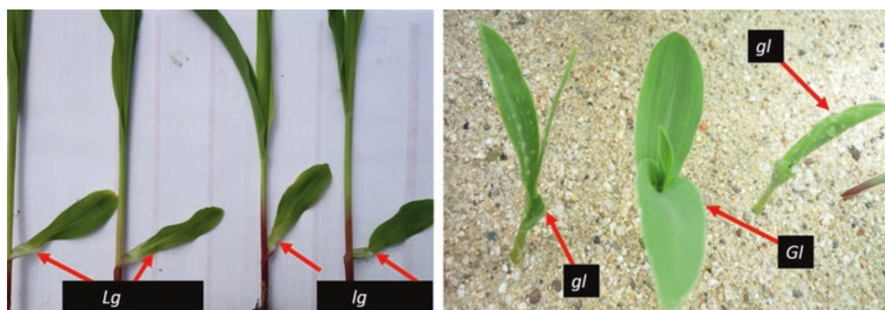

Fig. 11.4 Phenotypes of most commonly used recessive mutations for the accurate measurement of the haploid induction rate. (**a**) *Liguleless* and (**b**) *glossy*

crosses (Prigge et al. 2012a; Chaikam et al. 2018). If the objective is to integrate new marker traits for haploid identification, inducers can be crossed with noninducers with specific marker traits, followed by selection for the marker phenotype in earlier selfing and backcross progenies, followed by selection for the HIR in families fixed for marker expression.

A major activity in inducer development is accurate determination of the HIR in plants or families that result from the inducer × inducer or inducer × noninducer crosses. The *R1-nj* marker, which is commonly used for haploid identification, is not appropriate for this purpose because it leads to high misclassification rates, as described below. Also, it is not usable in inducer × noninducer crosses because it will segregate in early generations. Consequently, HIR measurement needs to be assessed by crossing the putative inducer candidates to any germplasm with recessive tester phenotypes because none of the morphological traits present in the inducers can be used for this purpose. Some of these recessive phenotypes include *lg2* (*liguleless*) conditioned by the *lg2* gene and *gl* (glossy) conditioned by the *gl* gene, which were demonstrated to aid the accurate determination of haploid induction rate (Fig. 11.4) (Melchinger et al. 2016b). In crosses involving inducers/inducer candidates with recessive testers, only the haploids show recessive phenotypes, thereby facilitating visual discrimination between haploids and diploids. Testers with the *lg* gene have been widely used to assess the HIR while developing new inducers (Lashermes and Beckert 1988; Röber et al. 2005; Prigge et al. 2012a; Melchinger et al. 2016b). Due to evidence that the inducer effect on the HIR is very significant and the tester and inducer × tester interaction is small, any tester that facilitates easy discrimination of haploids can be used for accurate determination of the HIR (Melchinger et al. 2016b). When assessing the HIR, sufficient numbers of testcross plants need to have their ploidy tested to be confident about the HIR measurement. In early generations, at least 200 testcross seeds should be planted and evaluated, while in advanced generations at least 1000 testcross seeds should be evaluated (Prigge et al. 2012a). The HIR should also be evaluated in multiple cycles and, if possible, in different environments to confirm the stability of the inducer line for high haploid induction capability. Even though the use of recessive testers

facilitates a precise assessment of the HIR, the process is resource intensive because it involves several steps like producing testcross seed and planting and evaluating the testcross seedlings for the phenotypes from many plants/families. As a result, it may limit the number of families to be tested for haploid induction capabilities.

Recently, an efficient method has been adapted to develop haploid inducers based on marker-assisted selection of the *qhir1* locus, which was shown to be critical for haploid induction and has significant effects on increasing the HIR (Dong et al. 2014; Chaikam et al. 2018). Marker-assisted selection (MAS) of *qhir1* facilitates the elimination of plants/families that do not have any capability of haploid induction or have a very low HIR (Dong et al. 2014; Chaikam et al. 2018). As discussed above, the *qhir1* locus is selected against due to segregation distortion, so MAS in early segregating generations results in increasing the frequency of genotypes that potentially have high HIR. Thus, MAS enables significant savings of the resources involved in testcrossing and evaluating the HIR of genotypes that may not have the capability to induce haploids. In addition to MAS, haploid-induction-associated traits can also be used to eliminate families that have no or low HIR. It has been shown that *qhir1* conditions the abortion of the endosperm and embryo in addition to haploid induction (Xu et al. 2013; Kelliher et al. 2017; Li et al. 2017; Nair et al. 2017). Indeed, almost all the ears from families with *qhir1* show high levels of abortion, whereas families without *qhir1* show no or extremely low abortion rates (Chaikam et al. 2018). Plants/families with endosperm and embryo abortion possess significantly higher HIR compared to families where endosperm and embryo abortion is not present. Further, families with high HIR have higher proportions of haploids in the progeny compared to families with no or low HIR. Among these HIR-associated traits, endosperm abortion can be easily scored on an intact ear with minimal expense and hence can be used to advance plants/families with or without MAS. Even though these traits can differentiate plants/families with and without haploid induction, they cannot differentiate families with different HIRs. Despite using MAS or endosperm abortion to select families with potentially high HIR, these plants/families still need to be evaluated based on testcrossing with recessive tester(s) to identify specific lines with highest HIR.

Maintenance of inducer lines also involves frequent testing of HIR using recessive testers because HIR can decrease drastically over the years if the inducer plants are not tested and selected for high HIR. Natural selection strongly disfavors the haploid induction trait (Melchinger et al. 2016b), and unless it is selected frequently, the trait can be lost from the inducers. The selective disadvantage of haploid induction results in segregation distortion (Barret et al. 2008; Prigge et al. 2011; Dong et al. 2013; Xu et al. 2013; Nair et al. 2017). Contamination of inducer stocks is the common cause of reductions in HIRs due to contaminant pollen having a higher selective advantage than inducer pollen. Rigorous scouting of inducer seed increase plots to eliminate plants that do not show inducer line characteristics is important. In seed increase plots, 25–50 desirable plants can be selected and testcrossed to recessive testers and also selfed. After evaluation of the testcrosses, seed from plants showing the highest HIR can be used for further line maintenance. This can be done in every cycle of inducer seed increase to ensure that the HIR is maintained in the

inducer lines. Agronomic traits such as pollen production, plant vigor, and resistance to important diseases should also be considered during maintenance breeding. Sib mating can be used instead of selfing during seed multiplication of inducers to avoid loss of vigor in the inducers (Chaikam 2012).

11.3 Identification of In Vivo Induced Maternal Haploids

Induction crosses typically result in 5–15% of the progeny being haploids. Thus, most of the seeds resulting from induction crosses are diploids and are not of any use in DH line production. Consequently, the identification of sufficient numbers of haploids for DH line production is a labor-intensive step that takes significant time. Haploids and diploids can be separated from each other at the seed, seedling, or adult plant stages. It is desirable to identify haploids at the seed stage because this can reduce the number of plants that require handling during the downstream stages of DH line production and therefore reduces production costs. Haploids and diploids can be separated based on the expression of certain genetic markers integrated into haploid inducers or based on contrasting characters in haploids and diploids.

11.3.1 Haploid Identification Using Genetic Markers

Dominantly expressed genetic markers manifesting their phenotype preferably at the seed or seedling stage can be incorporated into maternal haploid inducers to assist with haploid identification. Diploids resulting from induction cross are typically hybrids between the male inducer parent and the female source population and therefore have genomes from both the parents, while haploids inherit only the maternal chromosome complement. However, haploid and diploid seeds look alike morphologically, and thus it is not possible to assess the ploidy status visually. Undoubtedly, the expression of genetic markers is very useful to separate a haploid seed from a diploid seed. *R1-nj*, which is an anthocyanin marker that is dominantly expressed on the seed, is commonly used for haploid identification, and all of the recently reported haploid inducers are equipped with *R1-nj* (Chaikam and Prasanna 2012; Melchinger et al. 2013). *R1-nj* phenotypic expression requires the expression of several other genes involved in anthocyanin biosynthesis, such as *A1*, *A2*, *C2*, *Bz1*, *Bz2*, and *C1*, and all these genes need to be incorporated into the inducer. The *R1-nj* phenotype is generally called the "Navajo" phenotype and is characterized by purple or red coloration in the aleurone layer of the endosperm and scutellum of the embryo (Fig. 11.5a) (Nanda and Chase 1966; Greenblatt and Bock 1967). In induction crosses, diploids express the Navajo phenotype in both the endosperm and embryo, while a haploid seed expresses anthocyanin only in the endosperm, and this facilitates visual discrimination of haploids from diploids (Nanda and Chase 1966). To identify about 1000–2000 haploids, it is necessary to visually inspect

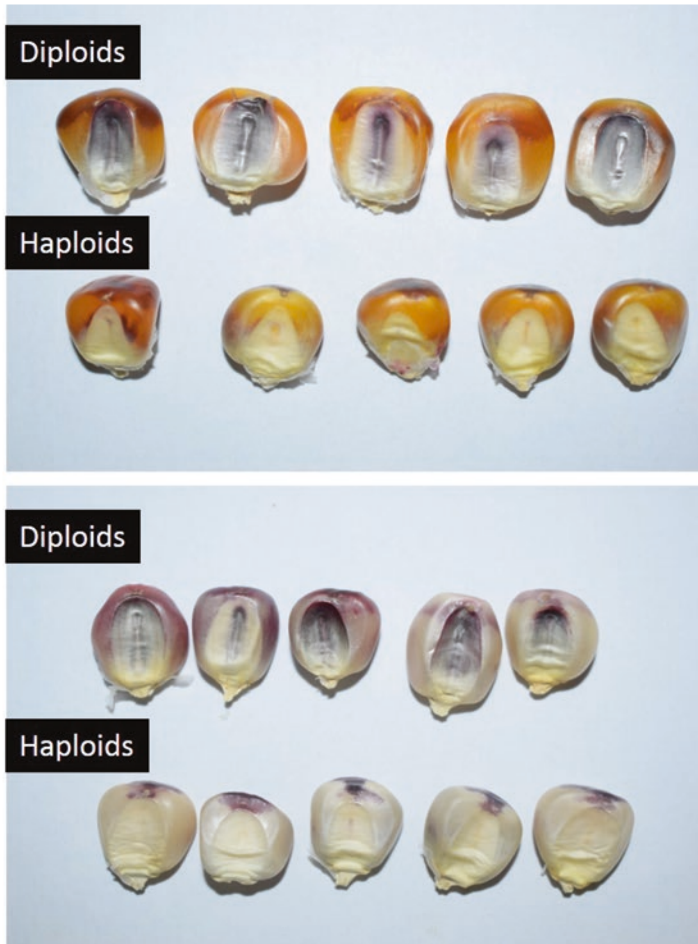

Fig. 11.5 (**a**) An *R1-nj*-marker-based haploid identification and (**b**) different levels of expression/inhibition of the *R1-nj* marker

approximately 10,000–20,000 seeds for the Navajo phenotype, which is a time-consuming and labor-intensive process. To address these issues, mechanical sorting is being optimized based on the *R1-nj* marker expression via multispectral (De La Fuente et al. 2017), hyperspectral (Wang et al. 2018), and fluorescence imaging technologies (Boote et al. 2016). However, these mechanical sorting technologies are not yet available for maize breeding programs.

Although the *R1-nj* marker is widely used, there are some practical problems limiting its use in different germplasm types. The major difficulty in using the *R1-nj* marker is inhibition of its expression due to anthocyanin inhibitor genes like *C1-I* (Fig. 11.5b). Indeed, *R1-nj* marker inhibition is a significant problem in tropical germplasm as ~30% of tropical elite inbred lines show complete inhibition (Chaikam

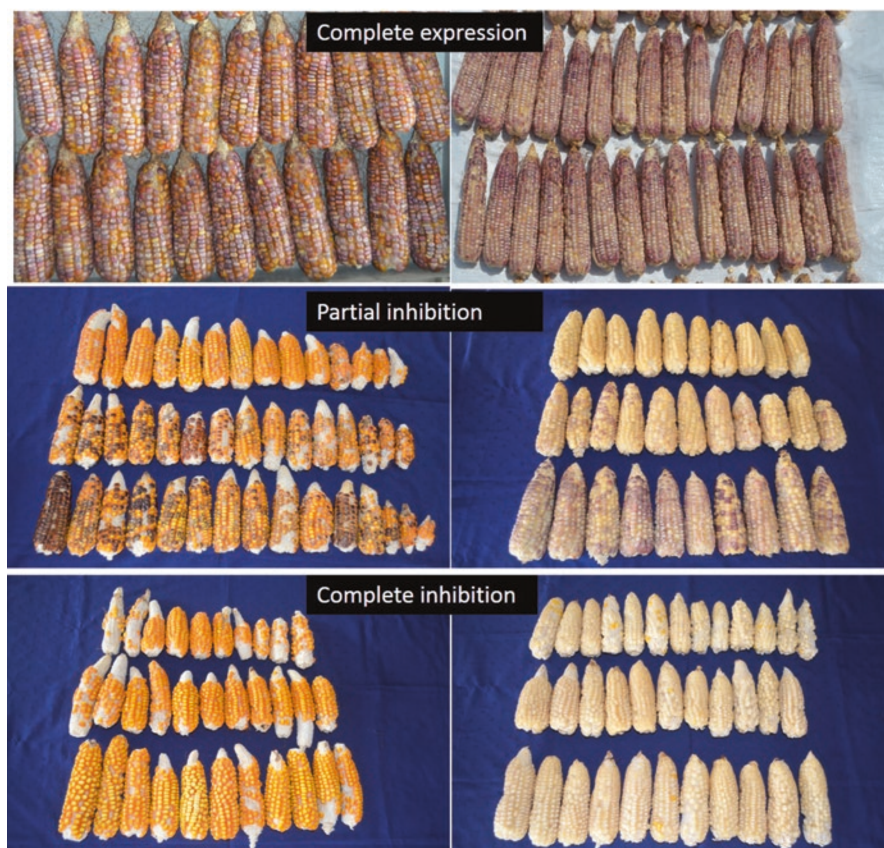

Fig. 11.5 (continued)

et al. 2015); thus, this marker is not suitable for identifying haploids in populations constituted from such inbreds. Inhibition of *R1-nj* marker expression is also common in temperate flint germplasm (Röber et al. 2005). In addition to complete inhibition, segregation of *R1-nj* marker expression also occurs in a significant proportion of tropical breeding populations and landraces, where only some proportion of seed resulting from induction crosses show the Navajo phenotype (Chaikam et al. 2015). In such cases, it is not possible to identify all haploids present in the induced seed. Using sequence variation in the *C1-I* gene, molecular marker assays were designed that could predict *R1-nj* marker inhibition or expression (Chaikam et al. 2015). Using these marker assays saves the costs involved in haploid induction and identification in populations with anthocyanin inhibitor genes. In addition to complete or partial inhibition, use of the *R1-nj* marker can also lead to high levels of false positives (Röber et al. 2005; Prigge et al. 2011; Melchinger et al. 2014; Chaikam et al. 2016) and false negatives (Röber et al. 2005; Chaikam et al. 2016). False negatives result in loss of valuable haploid seeds in the diploid fraction, and false positives

result in wastage of resources in downstream processes. One more problem with the *R1-nj* marker is that the Navajo phenotype can be masked in germplasm with natural purple or red anthocyanin coloration in the pericarp/endosperm of the seed. This coloration is common in many tropical maize landrace accessions, and so *R1-nj* is not effective for haploid identification in such germplasm (Chaikam et al. 2016). Considering these limitations, the integration of additional marker systems into haploid inducers and implementing other methods of haploid identification are necessary to increase the accuracy and efficiency of haploid identification.

Another genetic marker that can aid haploid identification at the seed stage and facilitate automation is based on high-oil xenia effects. Normal maize germplasm contains only about 3–4% oil, and most of it accumulates in the embryo (Preciado-Ortiz et al. 2013). Haploid seeds and diploid seeds naturally show differences in their average oil content, with diploids typically possessing 0.6–0.8% higher oil content than that of the haploid seeds (Rotarenco et al. 2007; Melchinger et al. 2014). Nonetheless, it was established that the haploids and diploids show a mixture distribution for oil content with a very big overlap. In addition, only small differences were observed for their mean oil content. Together, this makes it impossible to separate haploids and diploids based on innate difference in oil content (Melchinger et al. 2013, 2014). Hence, it is compulsory to employ haploid inducers with high-oil traits to distinguish haploids and diploids based on oil content (Melchinger et al. 2014). High oil trait was integrated in some inducers such as CAUHOI (with 7.8% oil content (OC)) (Chen and Song 2003), CHOIL (~8.5% OC) (Dong et al. 2014), UH600 (9.9% OC), and UH601 (11.6% OC) (Melchinger et al. 2013). Haploids resulting from the use of such inducers showed lower mean oil content than that of diploid seeds, but the mean oil content of both haploids and diploids depended on the oil content of the inducer and the source population. It was also concluded that inducers with at least 10% oil content is necessary to ensure a clear separation of haploids and diploids and control misclassification rates. Based on all these observations, a criterion was developed for the effective use of high-oil inducers with minimal misclassification rates (Melchinger et al. 2014). Completely automated high throughput platforms that show very high repeatability for oil content measurements and high accuracy of distinguishing haploids and diploids were recently developed that aid in the automated sorting of single seeds. These platforms are based on nuclear magnetic resonance (NMR) (Wang et al. 2016; Melchinger et al. 2017, 2018). In addition to enabling automation, the high-oil marker enables haploid identification in all germplasm types, including landraces and wild relatives like teosinte as this marker is genotype independent. The constraints of using high-oil-based haploid identification methods are availability of high-oil inducers and automation technology. Even when available, the initial cost of establishing the NMR-based automated platform could be high, limiting its immediate adaptation.

Several marker systems have been proposed to identify haploids at the seedling stage. Expression of anthocyanin coloration in root tissues occurs rarely in maize germplasm, and it is expressed in a dominant fashion (Chaikam et al. 2016). When inducers having a red root marker are used in induction crosses, only diploid

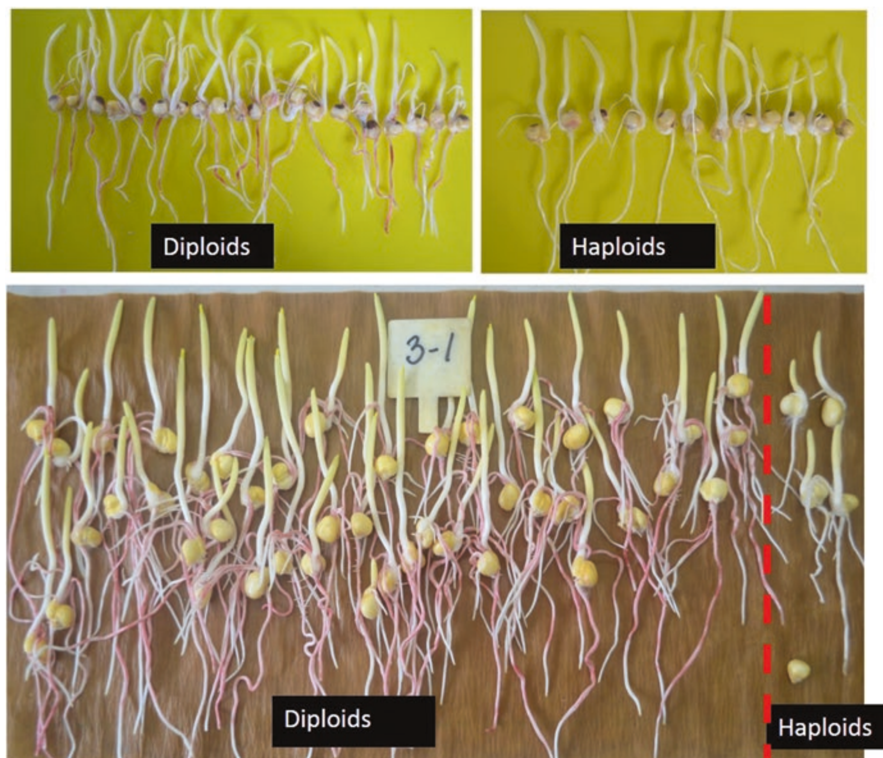

Fig. 11.6 Red-root-marker-based haploid identification

seedlings express red/purple coloration in roots, and so haploids with white roots can be easily separated (Fig. 11.6). Inducers combining *R1-nj* and red root markers have been developed (Rotarenco et al. 2010; Chaikam et al. 2016). Since red/purple root coloration in maize roots is very rare, it does not suffer from the anthocyanin masking that affects *R1-nj*, and hence it can be used in many landraces and (OPVs). The red root marker is also expressed independently of the *R1-nj* marker (Chaikam et al. 2016) and can therefore complement *R1-nj* for haploid identification in many different types of germplasm. However, the limitation to red root marker usage is the need to germinate large numbers of seeds to identify desirable numbers of haploid seedling, which would be labor intensive and would need considerable greenhouse space. Consequently, it would be appropriate to use the red root marker when the *R1-nj* marker is not suitable due to its complete or partial inhibition or masking of the Navajo phenotype by natural anthocyanin expression in seeds. Nevertheless, the false positives that result from *R1-nj*-based haploid identification can be effectively eliminated by using the red root marker.

The purple sheath marker was proposed to aid the elimination of false positives among putative haploids identified on the basis of the *R1-nj* marker and was

incorporated into several haploid inducers (Röber et al. 2005; Li et al. 2009; Prigge et al. 2012a). The major disadvantage of this marker is that it is expressed during late vegetative stages and is therefore not suitable for eliminating the false positives before chromosomal doubling treatments (Chaikam et al. 2016). The purple sheath phenotype has also been demonstrated at the high frequency of ~60% in tropical landraces and ~10% in elite CIMMYT inbreds naturally (Chaikam et al. 2016), and so it can be masked by natural anthocyanin coloration in the leaf sheaths. Considering these factors, this marker is of very little use in haploid identification or the elimination of false positives.

Transgenic markers can also be integrated into haploid inducers to aid in haploid identification. When 35S promoter-driven engineered green fluorescent protein (EGFP) was integrated into a temperate haploid inducer and used for induction crosses, EGFP expression was recorded in the endosperm, embryos, roots, and coleoptiles of emerging diploid seedlings (Yu and Birchler 2016). This study indicated that the EGFP marker could be used at the preemergence stage, at the postemergence stage, and in embryo rescue protocols but not for screening dry seeds. Another transgenic marker proposed for haploid identification incorporated BASTA herbicide resistance (Geiger et al. 1994). Use of the inducer equipped with the BASTA resistance gene results in diploid seedlings that are resistant to herbicide and haploids that are susceptible. Susceptibility can be tested by treating a portion of the terminal leaf without greatly damaging or killing the haploid seedlings. Just like a red root marker, this herbicide resistance marker also requires substantial labor to germinate and test thousands of seedlings. In addition, transgenic haploid inducers may not be usable in many countries due to restrictions on the use of transgenes.

11.3.2 Haploid Identification Based on Natural Differences in Haploids and Diploids

Haploids and diploids show variations in several characteristics owing to the differences in chromosome number. Such natural variations between haploids and diploids can be exploited to differentiate them. At the seed stage, haploids and diploids show differences in seed weight (Melchinger et al. 2014; Smelser et al. 2015), but the weight distributions of haploid and diploid seeds from the same population almost overlap (Melchinger et al. 2014), making seed weight an unreliable characteristic for haploid identification. At seedling stage, several traits like coleoptile length, radicle length, and the number of seminal roots diverge significantly between diploids and haploids. Haploid seedlings tend to show significantly lower values for all the traits (Chaikam et al. 2017). Hence, seedling traits can be used to separate haploids from diploids (Fig. 11.7). However, this will necessitate germinating and evaluating large numbers of induced seed. Because this could be resource intensive and costly, it would be pragmatic to use seedling traits when other methods will not work. Nevertheless, seedling traits can be effectively used to reduce the false positives generated with the *R1-nj* marker (Chaikam et al. 2017), and seedling traits can

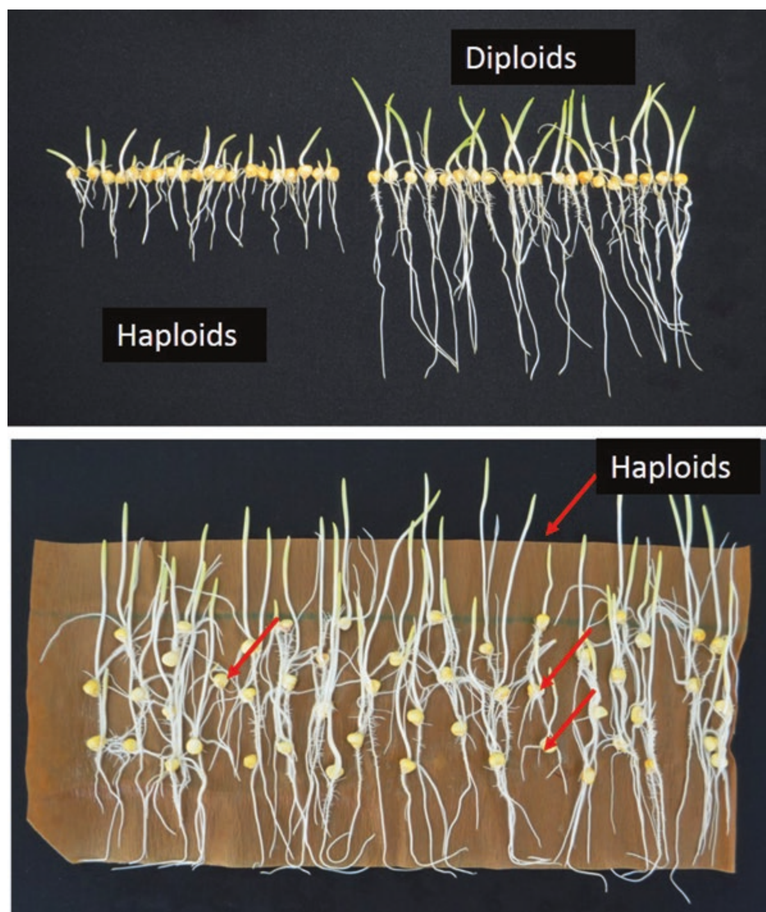

Fig. 11.7 Haploid identification based on seedling traits

potentially be used in combination with any other methods of haploid identification for the same purpose.

Stomatal sizes in haploids and diploids vary significantly and can be used to differentiate haploids and diploids (Choe et al. 2012). However, a more detailed analysis has indicated that the variance in the mean stomatal lengths of individual plants is large, making reliable differentiation of haploids and diploids difficult (Molenaar et al. 2019). It is also difficult to analyze stomatal length in a large number of seedlings, resulting from induction crosses in a high throughput manner, and so automation would be required to make this method efficient. Flow cytometry has also been assessed to differentiate haploids from diploids (Molenaar et al. 2019). This recent study revealed that flow cytometry cannot be used to distinguish haploids and diploid seedlings following chromosomal doubling, and the technique is also limited by the need for expensive equipment and a trained workforce, and it is currently not high throughput.

Maize haploid and diploid plants show distinct differences in many aspects during vegetative and reproductive growth. Haploids can be easily distinguished from diploids by their poor vigor; leaf characteristics like erect, narrow, and pale; and lack of pollen or poor pollen and seed production (Chase 1964b, 1969; Liu et al. 2017; Wu et al. 2017). However, the identification of haploids at the vegetative or reproductive stages is not a very efficient screening method because thousands of diploids must be maintained into maturity, which is costly. Most DH production pipelines do not rely on this method, but it could have some value for identifying any false positives that escape through earlier elimination steps.

In total, there are many prospects for increasing the efficiency and accuracy of haploid identification. For example, by developing and using an inducer with multiple marker systems like the high-oil, *R1-nj*, and red root markers, the majority of diploids can be discarded very quickly based on oil content in a high throughput manner, followed by the elimination of false positives using the *R1-nj* and red root markers. At CIMMYT, a haploid identification protocol that is first based on *R1-nj* marker expression at the seed stage and later based on the red root marker and seedling traits at the seedling stage is being implemented. This reduced the number of false positives that enter the treated haploid nursery from 15–40% to less than 5%, thus saving significant resources that would otherwise be expended on caring for plants that have no use in DH line production.

11.4 Doubling Haploid Genome

Haploid seeds or plants need to be subjected to chromosomal doubling as most haploids are sterile and do not produce pollen. However, most haploids (~97–100%) produce seeds when cross pollinated with pollen from normal diploid plants (Chalyk 1994; Geiger et al. 2006). So while female fertility in haploids is not a limitation in the production of DH lines, restoring fertility in the male reproductive parts is important for self-pollination and the production of seed (Chalyk 1994; Kleiber et al. 2012; Ren et al. 2017a, b; Wu et al. 2017). Sterility in haploids results from improper meiotic divisions due to lack of chromosomal pairs, which result in nonformation of functional gametes (Chaikam and Mahuku 2012). For this reason, the objective of chromosomal doubling processes is to ensure that homologous chromosomes can pair and that meiosis continues normally, resulting in the restoration of fertility. Chromosomal doubling can be achieved by treating haploids with certain chemicals or relying upon the spontaneous chromosomal doubling that is innate to certain maize genotypes.

11.4.1 Artificial Genome Doubling

For artificial chromosomal doubling of haploids, chemicals that exhibit antimitotic activity and reversibly arrest cell division are used. Colchicine, a chemical compound extracted from *Colchicum autumnale*, is most widely used for chromosomal

doubling in DH line production pipelines (Chaikam and Mahuku 2012; Melchinger et al. 2016a). Colchicine causes chromosomal doubling by binding to β-tubulin, arrests the formation of tubulin dimers, and prevents the formation of microtubules. The absence of microtubules during mitosis in the shoot apical meristematic cells prevents the detachment of replicated chromosomes, polar movement, and cell division, resulting in cells with double the number of chromosomes.

Standard chromosomal doubling protocols involve immersion of 4–5-day-old seedlings in a solution containing 0.04–0.06% colchicine and 0.5% DMSO for 8–12 h (Fig. 11.8) (Chaikam and Mahuku 2012; Prigge and Melchinger 2012). These protocols were originally described in the 1990s and are generally referred to as the seedling immersion method (Gayen et al. 1994; Deimling et al. 1997). These protocols involve germination of haploid seeds on paper towels for 96–120 h until the coleoptiles are about 2 cm long. The coleoptile tip is cut off before keeping the seedlings in the doubling solution to facilitate the uptake of doubling chemicals. Thereafter, seedlings are thoroughly washed under tap water to remove the doubling chemicals and allowed to recover in a greenhouse by potting them in peat moss in trays or biodegradable pots until the three-leaf stage. Fully recovered seedlings (10–15 days depending on the growth of the haploids) are transplanted in the field (Chaikam and Mahuku 2012). Depending on the germplasm, a success rate of 10–30% can be obtained using these protocols (Chaikam and Mahuku 2012; Melchinger et al. 2016a). However, colchicine is carcinogenic, and hence the handling and disposal of colchicine should follow very strict occupational health and safety standards (Chaikam and Mahuku 2012; Melchinger et al. 2016a). Consequently, optimizing chromosomal doubling protocols with less toxic alternatives is desirable (Chaikam and Mahuku 2012).

Fig. 11.8 Process of chromosomal doubling in putative haploids. (Reproduced important steps from Chaikam and Mahuku 2012)

Several herbicides are proposed as being antimitotic because they inhibit microtubule assembly or microtubule organization (https://hracglobal.com/tools/world-of-herbicides-map). Some of these herbicides have been tested as a less toxic alternative to colchicine for the chromosomal doubling of maize haploids. Three of these antimicrotubule herbicides—oryzalin, trifluralin, and flufenacet—have been shown to be not effective for chromosomal doubling in both in vitro (Wan et al. 1991) and in vivo DH production (Melchinger et al. 2016b). However, a combination of 0.5% dimethyl sulfoxide, 20 mg L^{-1} amiprofos-methyl (APM), and 4 mg L^{-1} pronamide applied in a seedling immersion method resulted in an overall success rate of chromosomal doubling closer to colchicine (Melchinger et al. 2016b). Oral LD 50 values for APM and pronamide indicate that they are several hundred times less toxic than is colchicine. Compared to colchicine-based protocols, antimitotic herbicides result in slightly lower chromosomal doubling efficiencies, but their use may minimize the occupational health risks and may eliminate costly disposal procedures.

Molenaar et al. (2018) tested nitrous oxide (N_2O) gas for chromosomal doubling because it is known to inhibit polymerization of microtubules (Kitamura et al. 2009). N_2O gas is relatively safe and has essentially no countereffects on health and is commonly used as an anesthetic and in food packaging. The procedure involves treating 3–4-day-old seedlings with N_2O gas in a pressure chamber. Calcium hydroxide is also placed in the chamber to remove carbon dioxide from respiring seedlings. The treatment lasts for 3 days at 25 °C at a pressure of 0.6 MPa. Following the treatment, seedlings are potted in a greenhouse and handled as described above for colchicine and herbicide treatments. This method showed similar success rates in chromosomal doubling as colchicine. Another advantage of using this protocol is that there is no need for sophisticated laboratory and chemical waste disposal facilities because N_2O may be released into the atmosphere in a well-ventilated area. However, this method requires a one-time investment in a pressure chamber that can withstand high pressures, which may increase the cost of initial establishment (Kato and Geiger 2002; Molenaar et al. 2018).

Several other treatment methods like application of colchicine to seeds (Gayen et al. 1994) and injection of colchicine into the shoot apical meristem (Zabirova et al. 1996) have been described. Higher success rates than that of the standard seedling dip treatment method were reported when 0.06% colchicine was applied to the seeds via a cut to a small portion of the plumule (Gayen et al. 1994). However, a similar experiment by Chalyk (2000) resulted in nonfertile plants. A more elaborate experiment at CIMMYT that tested various concentrations of colchicine for 5 h on seeds imbibed in water for 18 h did not result in any significant differences in chromosomal doubling rates compared to spontaneous chromosomal doubling (not published). Success in chromosomal doubling was also much lower than in the standard seedling immersion method when colchicine solution was injected 2–3 mm above shoot apical meristem (Chalyk 2000). Exposing the roots of 10–12-day-old seedlings to colchicine and mitotic herbicides was evaluated by Deimling et al. (1997) and Melchinger et al. (2016b), respectively, and the results indicated that this method was also much less efficient than seedling immersion treatments. Treating

adult maize plants with 0.6 MPa N_2O at the floral primordial stage (growth stages V3–V8) was also tested (Kato and Geiger 2002, Kato 2006). This method was effective in doubling the haploid genome but is not high throughput due to the need to grow haploid plants to adult stage in pots for treatment at the flowering stage and the requirement for larger pressurized chambers. Another method that is not yet tested is spraying of plants grown in the field, which has the potential to reduce the labor costs involved in greenhouse potting and filed transplanting the treated seedlings (Melchinger et al. 2016b).

11.4.2 Spontaneous Genome Doubling

As early as in 1949, it was observed that some haploid plants can produce pollen without any chemical treatment and upon self-pollination can produce seed for DH lines (Chase 1949). This phenomenon is generally referred to as spontaneous chromosomal doubling. The rate of spontaneous chromosome doubling may be calculated as the proportion of fertile plants (FP) setting seed among the total haploid plant population (Kleiber et al. 2012), which is similar to the overall success rate for artificial chromosome doubling. The FP in most maize germplasm studied is low, but significant differences were observed in FP among different germplasm types (Kleiber et al. 2012). This study also revealed that elite tropical germplasm had significantly higher FP values than tropical landraces had, although both of these germplasm have mean values ≤1%. FP ranged from 0% to 16.7% in intrapool crosses from Stiff Stalk, Lancaster, and Iodent heterotic groups, and heritability for FP was 0.79 in temperate crosses (Kleiber et al. 2012).

Spontaneous chromosomal doubling is mainly limited by haploid male fertility because the tassels do not produce pollen, but most haploid plants produce seed if pollinated with pollen from diploid plants. Spontaneous chromosome doubling generally results in only partial fertility as it results in chimeric tissues with both haploid and diploid cells. Consequently, pollen shedding anthers usually only emerge in limited sectors of a tassel. The proportion of plants with emerged anthers, which is scored as the anther emergence rate (AER), has been proposed as an effective measure of male fertility (Kleiber et al. 2012; Ren et al. 2017a; Wu et al. 2017). In diverse U.S. and Chinese germplasm, AER showed significant genetic variance (Wu et al. 2017). Heritabilities for AER are relatively high, ranging from 0.68 for inbreds to 0.78 for hybrids (Wu et al. 2017). At CIMMYT, a study of spontaneous fertility restoration by scoring haploid male fertility (proportion of total haploids producing pollen) and FP in haploids derived from 330 tropical inbred lines revealed high heritability and good variation for haploid fertility traits (Chaikam et al. 2019). Some of these tropical inbred lines showed an extraordinary capability for spontaneous fertility restoration showing the rates of FP that exceeds what is achieved using artificial chromosomal doubling methods. Such inbreds can be used as donors to improve spontaneous doubling rates in tropical maize germplasm. Together, several of these studies indicate that the rate of spontaneous chromosomal doubling is too low for

effectively using it in DH production. However, availability of greater genetic variation for spontaneous doubling, coupled with higher heritabilities, points to the possibility of improving the response to spontaneous chromosomal doubling by selection (Kleiber et al. 2012; Ren et al. 2017a; Wu et al. 2017; Ma et al. 2018). A recent study by Molenaar et al. (2019) revealed that the spontaneous chromosomal doubling rate could be increased from ~5% to 50% in two populations with three cycles of recurrent selection for haploid male fertility. A predominance of additive effects and some epistatic effects were found to underlie spontaneous chromosomal doubling.

Haploid male fertility has received a lot of attention recently in genetic studies due to the limitations of DH line production via spontaneous doubling. Wu et al. (2017) indicated that two or more major genes with additive effects condition spontaneous haploid male fertility. Several QTLs affecting haploid male fertility have been identified in mapping studies using biparental mapping populations derived from parents with low and high haploid male fertility (Ren et al. 2017a, b; Yang et al. 2019). Despite these advances on the genetic front, the causes of spontaneous chromosome doubling are still elusive. Several mechanisms like somatic cell fusion, endoreduplication, endomitosis, and nuclear restitution have been put forward as possible mechanisms (Jensen 1974; Shamina and Shatskaya 2011; Testillano et al. 2004).

11.5 Production of Seed for DH Lines

Handling haploid seedlings and plants is a delicate job as they are weak and can be easily damaged at various steps, like the chromosomal doubling process and recovery in the greenhouse and in the field. Haploids are also susceptible to various biotic and abiotic stresses (Mahuku 2012). Chemical toxicity during chromosomal doubling treatments imposes severe stress on haploids, increasing the mortality of the seedlings in the greenhouse and in the field. To reduce mortality during chromosomal doubling treatments, seedlings should be handled very carefully because the coleoptile is fragile and easily breaks if not handled properly. After chromosomal doubling treatments, the coleoptiles and roots become even more fragile, stiff, and easily broken. To avoid such damage, seedling should be handled by holding the seed.

Recovery of colchicine-treated seedlings can be achieved in a greenhouse or screenhouse with proper growing conditions. The greenhouse/screenhouse should be maintained at a temperature between 20 and 30 °C. Peat moss is an ideal medium for growing haploid seedlings. Haploid plants subjected to chromosomal doubling (D_0 plants) can be potted in thermocol or plastic trays or degradable jiffy pots. Potting medium should be watered sufficiently to avoid excess moisture, which can lead to fungal/bacterial infection on the seedlings. Soluble fertilizers can be applied in required doses with the irrigation water, and foliar fertilizers can also be applied for the quick recovery of seedlings. Fertilizers with high phosphorous aid in the

establishment of root systems and assist in speedy recovery (Mahuku 2012). Timely control of insect pests and diseases is also needed. Rats and birds can also cause severe damage to seedlings, and hence the greenhouse should be protected from these. Plants can be recovered up to the three-leaf stage (10–15 days) in the greenhouse and then transplanted into the field.

Transplanting the recovered seedlings into the field is another major stage at which maximum mortality can happen if not properly managed. Before transplanting, fields can be plowed into ridges and furrows. Planting haploids on the ridges helps to establish root systems due to the loosened soil. Before transplanting, the soil should be well saturated with water. Prior to moving the seedlings from the greenhouse to the field, the seedlings need to be well watered. Transplanting should be conducted preferably in the late afternoon to evening to avoid high temperatures in the middle of the day. Plants should be removed from their trays, taking care to avoid damage to the root system. In soils with high clay content, biodegradable jiffy pots may not degrade quickly. When transplanting in such soils, jiffy pots should be removed just before planting. It is also advisable to use shade nets in locations with high temperature and light intensity for at least a week for the effective recovery of transplanted seedlings.

Another major aspect of producing DH lines involves managing the putative DH plants under field conditions (Fig. 11.9). Since field conditions are difficult to control, selection of a proper site is very important for the optimal growth of D_0 plants. Locations with excessive heat and intensity of sunlight should be avoided. Locations with maximum daytime temperatures of 35 °C and more than 20 °C at night are ideal

Fig. 11.9 Management of D_0 seedlings and plants in the nursery

for a D_0 nursery. If the D_0 nursery must be in a location with high temperatures and high light intensity, the plants can be grown under shade nets to lower the incident radiation. Continuous use of shade nets throughout the growing period can result in very late flowering. To avoid such problems, shade nets may be used only during the middle of the day when temperatures are high. Another option is to use them only during critical phases, like seedling recovery and at the flowering stage. Application of fertilizers to the soil in recommended doses before transplanting and before flowering helps plants during vegetative and reproductive development. In addition, application of foliar fertilizers with micronutrients at 2–3-week intervals also aids in the proper establishment of D_0 plants and in ensuring good seed quantity and quality. Controlling insect pests and diseases should be given high priority by implementing a regimented crop protection program. Selecting a site that has no or minimum disease and insect pressure is desirable. In general, haploids show sensitivity to many insecticides and fungicides at recommended rates. Hence, any new chemicals should be first tested on a few haploids before applying them to the whole field. Insecticides and fungicides can cause tassel burning, and so applications should be stopped at least 1 week before tassel emergence. Weeds need to be controlled at young stages in particular. Haploids show sensitivity to postemergent herbicides, so physical controls like plastic mulch can be used to reduce weeds around the plants.

In the D_0nursery, identification and elimination of diploid plants that were misclassified as haploids are important to avoid competition with D_0 plants for nutrients, light, water, etc. They should be eliminated from the field before the flowering stage as they can cause pollen contamination. True haploid plants typically show poor vigor, erect leaves, and thin and paler leaves compared to diploids. If the inducers have purple color stalks, the false positives also show purple stalk coloration.

At the pollination stage, silks of D_0 plants should be covered with shoot bags to prevent pollen contamination. Haploid tassels need to be inspected regularly for any extruded anthers. Chromosomal doubling treatments may or may not lead to the complete doubling of the chromosomes of all cells of a seedling. As a consequence, the extent of tassel fertility varies widely, from entire tassels becoming fertile to only a few sections of the tassels becoming fertile, with only small numbers of anthers (Fig. 11.10). Sometimes even the extruded anthers can be dry and lack pollen. Any tassels with extruded and plump anthers can be covered with wax-coated glassine tassel bags or normal tassel bags in the evening or early in the morning, and pollen can be collected in the bags by shaking the tassels (Fig. 11.11). The tassel bags can be inspected for pollen shedding, and self-pollination can be conducted if pollen is present. To ensure good seed set, each pollen producing D_0 plant can be pollinated two or three times on consecutive days. Seed set on D_0 plants varies greatly with many of the ears having just one to only a few seeds (the seeds represent a completely homozygous line generally referred to as D_1). For example, an average of four seeds per D_1 ear was reported in a study by Kleiber et al. (2012). In CIMMYT DH production pipelines, it is common that 40–60% of DH lines have more than 25 seeds and the rest of the DH lines less than 25 seeds (not published). The ears with seeds can be harvested from D_0 plants at physiological maturity. The stored seeds require protection from ear rots, insect larvae, and weevils with the use

Fig. 11.10 Variation in tassel fertility in D_0 plants

Fig. 11.11 Self-pollination of fertile D_0plants

of appropriate insecticides and fungicides to ensure long-term seed health. DH line seeds can be directly planted for per se evaluations and testcrosses or can be used for molecular marker applications.

11.6 Benefits of Using DH Lines in Maize Breeding

The homozygous inbred lines needed for hybrid maize breeding and genetic studies can be developed on a fast track in 1 year using DH technology, which is in sharp contrast to the 3–4 years of inbreeding required with the conventional recurrent

selfing method that employs off-season nurseries. When DH, pedigree, and single-seed descent methods of inbred line development were compared under different testing regimes, including the conventional 3-year phenotyping, accelerated 2-year phenotyping, and single-year phenotyping with genomic selection, Atlin et al. (2017) showed a reduction in the overall breeding cycle time of at least 1 year compared to single-seed descent and 2 years compared to pedigree methods that use DH technology. Complete homozygosity of the DH lines makes them very suitable for variety registration/variety protection because they comply with the distinctness, uniformity, and stability (DUS) criteria, while the residual heterozygosity in conventional inbred lines can delay plant variety registration (Schmidt 2003; Röber et al. 2005; Geiger and Gordillo 2009). Considering this, the most significant economic advantage of using DH lines in maize breeding was noted as the reduced time to commercialization or the time to market (Seitz 2005; Bordes et al. 2006; Geiger 2009).

Another major advantage of using DH lines is simplification of logistics in breeding programs. Breeders develop thousands of segregating plants/families in early generations and hundreds of inbreds with various degrees of homozygosity at advanced generations from each population during conventional line development. When using DH lines, breeders circumvent various inbreeding cycles and get access to the desired number of completely homozygous inbred lines at once, eliminating the need for handling large numbers of plants or families from different stages of inbreeding. Thus, when using DH lines, operations like planting nurseries, line maintenance, shipping seed, managing inventories, etc. become much simpler (Röber et al. 2005; Prasanna 2012). Simplified logistics can lead to significant cost savings in maize breeding programs in the long run (Gordillo and Geiger 2008; Jumbo et al. 2011).

Compared to conventional inbred lines, higher selection gain is possible when using DH lines. DH lines allow more accurate phenotyping over locations and years, compared to inbred lines, with their varying levels of homozygosity (Yan et al. 2017). DH lines also exhibit maximum genetic variance compared to F_2 plants or selfed families (F_3, F_4 etc.) (Bordes et al. 2007; Gallais and Bordes 2007; Mayor and Bernardo 2009). Higher genetic variance among the DH lines results in higher heritabilities for various traits in the per se selection and in testcross evaluations compared to conventional inbred lines. Higher genetic variance and increased heritabilities result in better response to selection. Increased heritabilities and decreased cycle time when using DH lines can result in greater genetic gain per year in maize breeding programs (Longin 2008; Sleper and Bernardo 2016).

DH lines are ideally suited for many molecular marker applications due to their complete homozygosity and 1:1 genotypic segregation of both dominant and codominant markers (Yan et al. 2017). Use of DH lines results in higher phenotype to genotype correlation, thereby facilitating better estimation of marker/QTL effects in marker trait association studies (Hyne et al. 1995). DH techniques in combination with marker-assisted selection allow faster and more efficient fixation of favorable

alleles. The combination of molecular markers and DH lines is a very powerful tool for target gene fixation and transgene stacking because the number of plants or families to be sampled for homozygote identification is severalfold lower compared to traditional methods, and this then saves significant costs in sample collection, DNA extraction, genotyping, and data analysis (Lübberstedt and Frei 2012). For example, to identify one genotype with five loci fixed in a homozygous condition using F_3 families, a minimum sample size of 1024 progeny needs to be evaluated compared to 32 DH lines (Ren et al. 2017b). In addition, DH lines show superior response to selection compared to F_2 populations when using marker-assisted recurrent selection and genome-wide selection, especially when dealing with complex traits and lesser sample sizes (Mayor and Bernardo 2009).

DH technology can also be used effectively to exploit the genetic variation present in landraces and open-pollinated varieties, which are not so amenable for inbreeding due to a high genetic load of deleterious alleles (Wilde et al. 2010). Such deleterious alleles are readily expressed in the haploid stage (Fig. 11.12) and hence can be purged before inbred line development occurs in the DH process. High heterogeneity in landraces and OPVs makes it difficult to evaluate them with high precision in replicated trials (Strigens et al. 2013). In contrast, DH lines from landraces and OPVs can be evaluated in replicated trials with high precision. Together, DH technology opens new avenues for utilizing the broader genetic diversity present in maize germplasm that were previously not accessed by maize breeding programs (Wilde et al. 2010; Strigens et al. 2013). In addition, landrace-derived DH lines are also well suited for genetic studies and association mapping due to low population structure and quick decay of linkage disequilibrium (Wilde et al. 2010; Strigens et al. 2013).

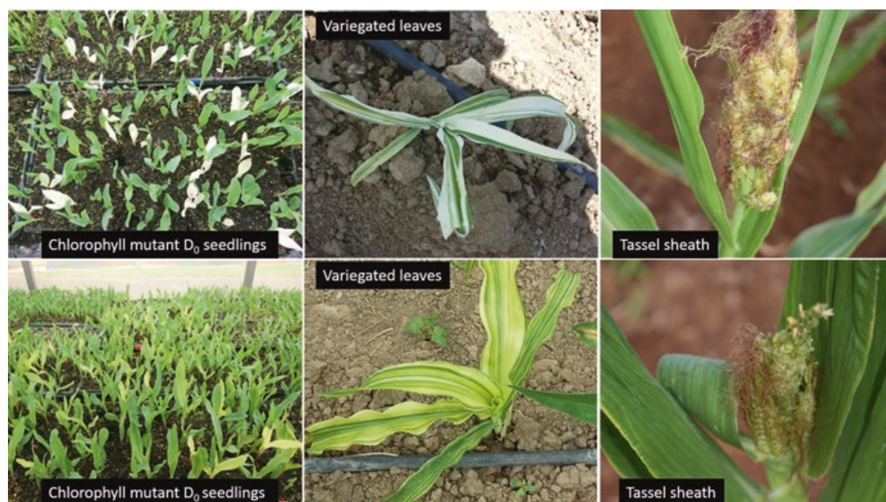

Fig. 11.12 Common mutations observed at haploid stage

11.7 Conclusions

Due to the multitude of advantages of using DH lines and the availability of protocols for efficient DH line production, DH technology is being widely adapted in maize breeding programs worldwide. However, until recently, DH technology was used exclusively for recycling elite germplasm when developing inbred lines for hybrid maize breeding. Recently it has been shown that DH technology can be a very powerful tool for tapping into landrace traits (Böhm et al. 2017; Melchinger et al. 2018), offering new opportunities for maize breeders to introduce novel variation and to expand the genetic base of elite germplasm. Reduced sample sizes and increased precision in estimating marker effects in DH lines will favor the integration of both DH and marker technologies in breeding programs to enhance breeding efficiencies. Refining DH technology to increase the efficiency of DH line production and reducing the cost per DH line are needed for the seamless integration of DH technology in maize breeding. These refinements can be targeted at different steps in DH line production. Improvements at the haploid induction stage can be achieved by developing highly efficient haploid inducers that induces haploids at very high frequency and perform under specific agroclimatic conditions. MAS for two major loci controlling haploid induction may enable the development of improved inducers with HIRs that exceed 20%. Automation of the haploid identification process using different phenotypic markers should also enable significant time and cost savings. Reducing the numbers of false positives during haploid identification via haploid inducers equipped with multiple marker systems could lead to further cost savings. Further efficiencies could be achieved via improved chromosomal doubling rates that should result in handling smaller numbers of D_0 plants and consequently lower labor requirements for their care. In addition, chromosomal doubling using nonhazardous chemicals or spontaneous doubling will simplify the production of DH lines and improve user safety. Optimization of conditions for seedling recovery after doubling treatments and field growing conditions of D_0 plants may improve the quantity and quality of DH line seeds, and in turn this may eliminate the need for D_1 line seed-increase step. Altogether, there are numerous opportunities for the further refinement of DH line production that may enable the adoption of more efficient breeding schemes.

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Chapter 12
Biofortification of Maize Using Accelerated Breeding Tools

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12.1 Introduction

Maize (*Zea mays* L.) is the most important cereal crop worldwide after wheat and rice with the global production of 1060 million tons (FAOSTAT 2017). It assumes its worldwide significance owing to utilization as a food of humans, livestock and poultry feed, and raw material for industrial and processed food products (Gupta et al. 2015). Maize alone provides over 20% of total calories in human diets in 21 countries and over 30% in 12 countries that are home to more than 310 million people. It provides around 62% of the proteins from all cereals in Mesoamerica, 43% in Eastern and Southern Africa, 28% in Andean Region, 22% in West and Central Africa, and 4% in South Asia (Hossain et al. 2018). Malnutrition and hidden hunger are the major problems of underdeveloped and developing nations of the world (Bouis and Saltzman 2017). Considering the importance of maize as food and feed, biofortification of maize including improvement in the protein quality, enhancement of provitamin A, and enrichment of micronutrients like Fe and Zn in the grain assumes great significance.

Biofortification involves enriching edible parts of food crops with essential nutrients by making them capable of producing or enhancing their capacity to produce those nutrients which they are deficient in through incorporation of suitable allele(s)/gene(s) in them. Biofortification of crops is also achieved by disabling them or reducing their ability to produce antinutritional factors that lower their biological value and/or limit bioavailability of nutrients contained in them. Maize has been biofortified for a range of essential nutrients that include tryptophan, lysine, provitamin A, iron, and zinc, among others (Gupta et al. 2015). Enhanced bioavailability of minerals in maize has been achieved by reducing phytate content in seed. Biofortification in maize has involved the utilization of various tools ranging from

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conventional breeding to genetic engineering through various molecular breeding approaches and various combinations thereof.

Modern molecular breeding tools like quantitative trait loci (QTL) mapping and association and genome-wide association studies (GWAS) enable the discovery of important genes and loci controlling the nutritional quality traits (Gore et al. 2009; Chia et al. 2012). These novel QTLs and single nucleotide polymorphism (SNP) loci are further utilized for the molecular marker development which leads to their direct utilization in the breeding programs through marker-assisted selection. The molecular markers enable breeders to identify the best parental inbred lines to perform marker-assisted backcrossing (MABC)—a method that involves the repeated crossing with the high yielding parent so that only the desired genes get transferred (Hossain et al. 2018). This approach cuts a year or 2 from 5 years required for developing a new variety. Further, the desired loci can be cloned and deployed through a genetic engineering tool. Doubled haploid breeding technology allows the isolation of novel recombinants and inbred lines along with cutting short of breeding cycle (Prasanna et al. 2012). Doubled haploid (DH) technology speeds up the breeding programs in comparison to the conventional methods.

Biofortification has its own share of challenges—genotype specificity, variable expression, and yield penalty being the major ones. Doubled haploids in conjunction with marker-assisted selection and gene editing tools have opened up vast opportunities for overcoming these challenges.

12.2 Marker-Assisted Selection/Marker-Assisted Backcross Breeding

Developing biofortified maize through conventional breeding is time- and resource-consuming even for simply inherited traits such as high tryptophan, high provitamin A, and low phytate on account of their recessive nature and need for biochemical evaluation during the segregating generations. Availability of co-dominant gene-linked/gene-based markers that enable sorting of recessive homozygotes in the segregating population has made marker-assisted selection/marker-assisted backcrossing a method of choice for generating biofortified inbreds and/or conversion of normal corn genotypes into their biofortified versions.

12.2.1 Tryptophan and Lysine

Tryptophan and lysine were among the first traits to be used for maize biofortification, and work toward enhancing the level of these essential amino acids in maize endosperm began with the discovery of high tryptophan mutant opaque-2. Opaque-2 mutants possessed around twice the level of these amino acids compared to normal

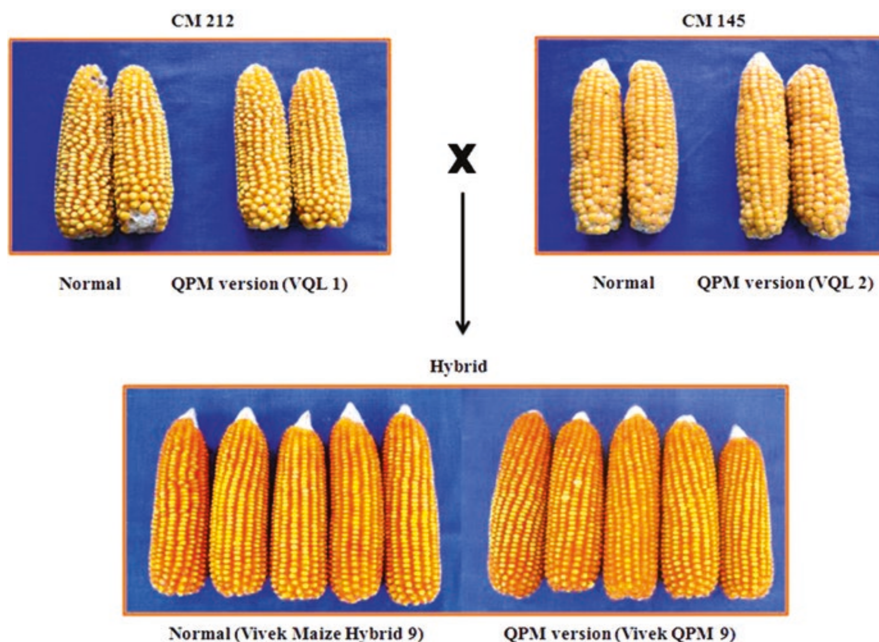

Fig. 12.1 MAS-derived QPM version (Vivek QPM 9) of normal maize Vivek Maize Hybrid 9

corn. QPM protein is comparable to milk protein in biological value (Vivek et al. 2008). The task was made relatively easier by relatively simple inheritance of the trait which is governed by a single recessive gene though later background modifier genes were found to considerably influence the endosperm texture. Use of marker-assisted selection for development/conversion of lines has resulted in the rapid development of high yielding maize hybrids with high tryptophan and lysine levels.

Babu et al. (2005) used a two-generation marker-based backcross breeding method in combination with phenotypic selection to develop high tryptophan version of normal corn inbred V 25. The first marker-assisted development of a QPM hybrid involved conversion of parental inbreds of hybrid Vivek Maize Hybrid 9 into their QPM versions and reconstitution of the hybrid as Vivek QPM 9 (Gupta et al. 2009). Using marker-assisted selection (MAS), they were able to recover inbred lines homozygous for o2 gene with more than 90–95% recurrent parent genome in a short span of 3 years, cutting the conversion time by about one-third when compared to conventional backcross breeding for a recessive gene (Fig. 12.1). Vivek QPM 9 possessed 0.83% tryptophan and 4.19% protein compared to 0.59% and 3.25% lysine in the original non-QPM hybrid. The yield of Vivek QPM 9 was at par with Vivek Maize Hybrid 9, and the level of resistance to turcicum leaf blight was also similar. Vivek QPM 9 was released for its commercial cultivation in India in 2008. The successful development of Vivek QPM 9 using MAS led to the conversion of many other popular hybrids into their QPM versions in the country. Notable among these are HM 4, HM 8, HM 9, HM 10, and HM 11 (Hossain et al. 2018);

DMH 117 from ANGRAU, Hyderabad (Surender et al. 2017); and Palam Sankar Makka 2 from CSKHPKV, Bajaura—with many others in the pipeline. Besides conversion of non-QPM hybrids into QPM hybrids, new QPM hybrids have been developed using QPM lines derived using MAS.

Opaque2 allele is most often accompanied by softer grain texture which is not a commercially desirable attribute. A new allele opaque16 reported by Yang et al. (2005) has been found to enhance lysine (Zhang et al. 2010, 2013) and tryptophan content (Sarika et al. 2017). Sarika et al. (2018) introgressed *o16* in parents of four commercial hybrids. The o2o2/o16o16 lines possessed up to 76% and 91% higher lysine and tryptophan compared to the o2o2 recurrent parents. The hybrids reconstituted were similar to their original versions in terms of yield and attributing traits. O16 mutant is also reported to have an insignificant impact on kernel modification and hardness (Sarika et al. 2018) implying that use of opaque16 as QPM donor would obviate the need for additional backcrosses required many a time to assemble background modifier genes to impart desired kernel quality to the QPM genotypes. Elimination of additional steps in the process will lead to resource and time saving and enhancement in efficiency of QPM breeding programs.

12.2.2 Provitamin A

The carotenoid biosynthesis pathway involves three major genes—psy1, lcyE, and crtRB1. Babu et al. (2013) revealed the significant interaction effect of crtRB1 and lcyE for proA accumulation in tropical maize. Favorable and positive interaction of crtRB1 and lcyE for proA was reported by Zunjare et al. (2017a) and Gebremeskel et al. (2018). A multiplex protocol has been developed for simultaneous foreground selection of lcyE and crtRB1 in the marker-assisted pyramiding program, which successively further advances proA biofortification program by saving significant cost and time (Zunjare et al. 2017b). Point mutations have been identified in lcyE (Zunjare et al. 2018a) and crtRB1 (Vignesh et al. 2013) which can be employed for development of cleaved amplified polymorphic sequence (CAPS) marker for their potent utilization in MAS (Zunjare et al. 2018a). Zunjare et al. (2018b) stacked parental inbreds of four popular maize hybrids with crtRB1, lcyE, and o2. The proA content in the introgressed progenies ranged from 7.38 to 13.59 μg/g compared to 1.65–2.04 μg/g in the recurrent parents. The reconstituted hybrids showed proA in the range of 9.25–12.88 μg/g which was 4.5–5 times higher than the original hybrids, while lysine and tryptophan content and yield were comparable to the original hybrids.

Naqvi et al. (2009) generated transgenic maize Carolight® by transforming a white endosperm corn variety M37W with two carotenogenic genes: Zmpsy1 (corn phytoene synthase 1) and PacrtI (bacterial phytoene desaturase). The endosperm of Carolight® acumulates 169-fold more β-carotene (provitamin A) as well as other nutritionally important carotenoids such as lycopene, lutein, and zeaxanthin.

12.2.3 Methionine

While tryptophan and related genes have been comprehensively studied and marker-assisted selection/backcrossing protocol has been well established, the genetics of methionine is poorly understood. Scott et al. (2004) reported that on an average, germplasm with the recessive o2/o2 mutation had reduced methionine levels than the O2/O2 (wild type) germplasm despite kernel hardness. This indicates that methionine levels could be reduced by the o2/o2 mutation, which makes the process of simultaneous increase in the levels of the two essential amino acids more complicated.

Variation in methionine levels in maize genotypes is ascribed to differential accumulation of Dzs10, a high-methionine seed storage protein, during maize endosperm development. The post-transcriptional regulation of Dzs10, which is on chromosome 9, is under a trans-acting process linked to dzr1 locus on chromosome 4 (Benner et al. 1989). Lai and Messing (2002) developed transgenic seeds by replacing cis-acting site for Dzs10 regulation. The transgenic plants expressed stable high levels of Dzs10 protein over five backcross generations of the transgene. The work led to a patented QPM superior in protein content with high lysine and methionine levels besides vitreous kernels suitable for storage and transportation. The QPM showed no yield loss compared to non-transgenic parent line and led to rapid weight gain in chicken when used in feed formulation without methionine supplementation.

Deng et al. (2017) ascribed the variation in methionine level in inbred lines to a number of quantitative trait loci (QTLs), none of which has been cloned yet. Newell et al. (2014) developed high and low methionine populations by recurrent selection for eight generations. Significant differences in methionine-rich seed storage protein genes were observed in comparison of low and high methionine populations, and two genes involved in sulfur acclimation. Cys2 and CgS1 showed considerable dissimilarities in allele frequencies when two selected populations were compared to the starting populations. High-stringency association analysis identified major genes that included dzs18, wx, dzs10, and zp27 across cycles of selection.

Serine acetyltransferase (SAT) is a key control point for S-assimilation leading to Cys and Met biosynthesis, and SAT overexpression is known to amplify S-assimilation without negative influence on plant growth (Tsakraklides et al. 2002; Martin et al. 2005). Xiang et al. (2018) overexpressed *Arabidopsis thaliana* AtSAT1 in maize under the control of leaf bundle sheath cell-specific rbcS1 promoter. The transgenic events exhibited up to 12-fold higher SAT activity without a negative impact on growth. The elite event with the highest expression of AtSAT1 showed 1.40-fold increase in kernel methionine. When fed to chickens, transgenic AtSAT1 kernels significantly increased growth rate compared with the parent maize line.

12.2.4 Phytic Acid

Phytic acid (myo-inositol-1,2,3,4,5,6-hexakisphosphate or Ins P6) typically represents approximately 75–80% of maize (*Zea mays*) seed total P. During seed germination, the phytate salts are broken down by the activity of phytases, releasing their P, mineral, and myo-inositol content for use by the growing seedling (Raboy et al. 2000, 2001). Phytic acid is an antinutritional factor, for it chelates mineral nutrients (manganese, calcium, iron, zinc, magnesium, etc.) and provides minerals in phytate non-bioavailable to humans and supplies phosphorus in phytate non-bioavailable to monogastric animals such as poultry, swine, and fish (Brinch-Pedersen et al. 2002). Moreover, PA is an environmental pollutant; the phosphorus unleashed from undigested PA excreted by monogastric animals can cause phosphorus pollution. Development of varieties with low phytic acid levels, therefore, is important from nutrition as well as environmental perspectives.

So far, in maize, three low-PA (lpa) mutants have been isolated, viz., lpa1, lpa2, and lpa3. Compared with wild-type kernels, the lpa1, lpa2-1, and lpa3 mutations achieved 66%, 50%, and 50% reduction in phytic acid content, respectively (Raboy 2002; Shi et al. 2005). *lpa*3 gene encodes *myo*-inositol kinase which can also produce glucose-6-P to Ins(3)P1. *lpa*2 encodes an inositol phosphate kinase that along with other kinases leads to phytic acid synthesis. The ABC transmembrane transporter encoded by *lpa*1 is involved in phytic acid transport into protein storage vacuole (PSV). The biosynthesis pathway ultimately leads to accumulation of phytic acid in the protein storage vacuoles (PSV) of maize seed (Fig. 12.2). The mutant alleles have been introgressed into elite inbred lines using marker assisted backcrossing (MABB). Sureshkumar (2014) used low phytate mutant Lpa2 as a donor for transferring lpa2 allele to elite inbred line UMI 395 using linked marker umc2230 for marker-assisted backcrossing. The phytic acid in the introgressed recurrent parent was also reduced to 1.73 mg/g compared to 2.6 mg/g in the original line. Mutant alleles lpa1 and lpa 2 alleles were introgressed into inbred lines CM 145 and V 334, respectively, at ICAR-VPKAS, Almora (Unpublished). The introgressed lines showed 25–30% reduced phytic acid with normal seed germination and plant vigor.

Transgenic approach has also been used to develop low phytate maize. Aluru et al. (2011) used the low phytic acid 1-1 (lpa1-1) mutant of maize to generate transgenic plants with up to 70 μg/g seed iron through the endosperm-specific overexpression of soybean ferritin which resulted in more than twofold improvement in iron bioavailability. Gene expression studies unveil a large induction of the YS1 transporter in leaves and severe repression of an iron acquisition gene DMAS1 in roots, suggesting significant alterations in the iron homeostatic mechanisms in transgenic lpa1-1. Chen et al. (2008) overexpressed *Aspergillus niger* phyA2 gene in maize seeds using a construct driven by the maize embryo-specific globulin-1 promoter. Phytase activity in transgenic maize seeds reached approximately 2200 units/kg seed, about a 50-fold increase compared to non-transgenic maize seeds. The phytase expression was reported to be stable across four generations. Drakakaki et al. (2005) generated transgenic maize plants expressing *Aspergillus* phytase both alone and in combination with the iron-binding protein ferritin.

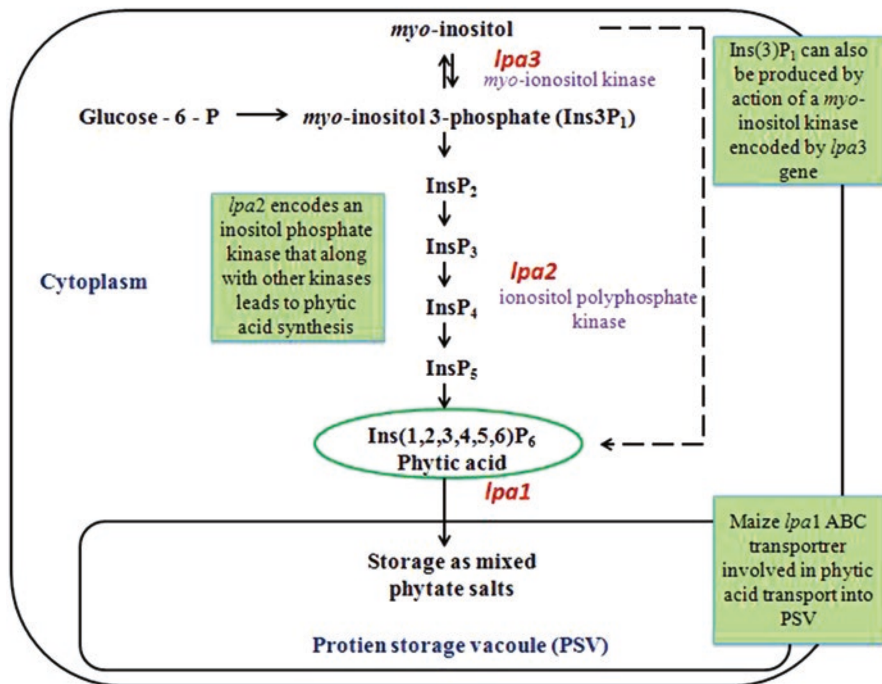

Fig. 12.2 Schematic model of phytic acid biosynthesis pathway

Maize seeds expressing recombinant phytase revealed enzymatic activities of up to 3 IU/g of seed. In flour paste prepared from these seeds, up to 95% of the endogenous phytic acid was degraded, with a collateral increase in the amount of available phosphate. In seeds expressing ferritin in addition to phytase, the total iron content was significantly increased. Shen et al. (2008) transformed maize inbred line 18-599 (red) with phyA gene using pCBA plant endosparm special vector. Phytase activities of dry seeds of three plants manifested a clear-cut increase compared to the control. The plant with highest phytase activity showed increase by 60.85% resulting in 47.84% increase in inorganic phosphate content. Alternatively, phytic acid has been lowered by expressing a fungal phytase resulting in threefold more bioavailable iron in maize (Drakakaki et al. 2005).

12.2.5 Iron and Zinc

Among the mineral nutrients required by humans for their well-being, Fe and Zn play vital roles in numerous metabolic processes and are required in trace amounts by plants as well as animals (Welch and Graham 2004). The complex mechanisms involved in the uptake, transportation, assimilation, and utilization of these mineral

nutrients in the plants place them among least understood processes. The intricate network of biosynthetic pathways comprising several genes and high environmental sensitivity makes identification and use of molecular markers for making rapid gains difficult. Consequently, the progress in breeding for Fe and Zn has remained very limited compared to other relatively simply inherited components of biofortification such as lysine, tryptophan, provitamin A, and phytate.

Qin et al. (2012) identified QTLs for Fe and Zn co-localized on chromosomes 2, 7, and 9 suggesting that amount of these minerals could be enhanced simultaneously by targeting the same chromosomal regions through marker-assisted selection. Šimić et al. (2009) reported a co-localization of three QTLs on chromosome 3 for Fe/P, Zn/P, and Mg/P ratios and overlapping chromosome regions of four QTLs on chromosome 6 for Fe/P ratio and P, Fe, and Mg concentration. One QTL on chromosome 5 that accounted for over 16% of the variation in grain iron concentration has been mapped by Jin et al. (2013). Two QTLs, *qFe5* and *qZn5*, located in the marker interval umc1429–umc1060 on chromosome 5 were also identified. Hindu et al. (2018) used GWAS on 923 maize inbred lines and identified 20 SNPs significantly associated with high kernel Zn concentration and 26 SNPs significantly associated with high Fe concentration. Of these, 11 SNPs for Zn and 11 SNPs for Fe had significant effects on the variance of these traits. These findings could be very useful for mineral biofortification of maize and may also facilitate the cloning of important genes in the background of these traits (Hindu et al. 2018). Since both high iron and high zinc are desirable traits for crops, the combined QTLs are helpful in breeding programs (Connorton and Balk 2019).

In addition to QTLs, various metal transporter genes such as ferritins, yellow stripe-like (YSL) transporters, natural resistance-associated macrophage protein (NRAMP), zinc-regulated transporters (ZRT), and iron-regulated transporter (IRT)-like proteins (ZIP) are reported to be involved in Fe-Zn absorption, mobilization, and redistribution in the maize kernels (Grotz et al. 1998; Vert et al. 2001; Waters et al. 2006). Candidate gene-based markers (SNPs and SSRs) for high iron and zinc transporter sequences are reported in maize (Sharma and Chauhan 2008), which can be utilized for identification of inbred lines with high Fe and Zn accumulation.

Kanobe et al. (2013) observed significantly higher concentrations of calcium, magnesium, and iron in maize transformed with soybean ferritin transgene (SoyFer1, M64337) and suggested that the soybean ferritin transgene affected the expression of native iron homeostasis genes in the maize plant. ZmZIP5 is a member of the ZIP transporter family, which has been shown to compensate for the growth defects of the yeast zrt1zrt2 and fet3fet4 double mutant (Li et al. 2013). ZmZIP5 was constitutively overexpressed in maize line Ubi-ZmZIP5. High levels of Fe and Zn were found in the roots and shoots, but decreased levels were found in the seeds. Use of legumin 1, an endosperm-specific promoter, to control ZmZIP5 expression resulted in an increase in Zn and Fe concentrations in the endosperm, implying that ZmZIP5 had an important role in Zn and Fe uptake and root-to-shoot translocation and endosperm-specific ZmZIP5 overexpression could be useful for Zn and Fe biofortification of cereal grains (Li et al. 2017).

12.3 Doubled Haploid (DH) Technology

Conventional plant breeding typically takes six to eight generations for the development of homozygous lines. DH technology has emerged as a robust tool for rapid generation of completely homozygous lines for accelerating hybrid development process in maize. Doubled haploid (DH) technology allows the generation of a large number of completely homozygous lines in two to three generations, thereby reducing the line development duration by more than half. The current method of DH development in maize involves in vivo production of haploids using *R1-nj* based haploid inducer lines. The inducer lines upon use as male render a small fraction of seed in the female ears haploid (Prasanna et al. 2012). The haploid seeds are subsequently diploidized to obtain completely homozygous lines. Identification of haploids at kernel stage is based on phenotypic expression of *R1-nj* ('Navajo' phenotype), which is characterized by purple coloration in the aleurone layer on the crown region of the endosperm and the scutellum of the embryo (Nanda and Chase 1966; Greenblatt and Bock 1967). The first-generation tropically adapted inducer lines developed by CIMMYT and the University of Hohenheim have high haploid induction capacity (~8–10%) (Prasanna et al. 2012). The doubled haploid production efficiency using CIMMYT's protocol is about 2–4%. Compared to first-generation haploid inducer lines, the second-generation haploid inducer lines (CIM2GTAILs) have higher haploid induction rates (~8–15%) under CIMMYT-tested (sub)tropical conditions in Mexico and Kenya in addition to better agronomic performance in terms of plant vigor, better standability, synchrony with tropical source populations, and resistance to important tropical foliar diseases and ear rots (CIMMYT 2017). DH technology increases genetic gain per cycle, increases efficiency of the breeding program, and reduces costs associated with research and development (Pioneer 2017). Owing to obvious advantage of DH technology over conventional breeding, the breeding operations of several large multinational seed companies are currently based on the use of DH lines for majority of breeding activities. During 2011, Pioneer had reportedly generated more DH lines than the total number of inbreds generated in the first 80 years of their breeding efforts (Prasanna et al. 2012).

Strategies to combine DH with marker-assisted selection have been proposed to accelerate approach to achieving target genotypes (Prasanna et al. 2012). In conjunction with molecular marker-assisted selection, DH technology can be effectively employed for developing multi-trait biofortified lines and hybrids in a shorter period of time. DH technology has been used for development of lines with *opaque2* allele (unpublished). QPM hybrid Vivek QPM 9 was used as source population for development of doubled haploid lines with *opaque2* alleles (Fig. 12.3).

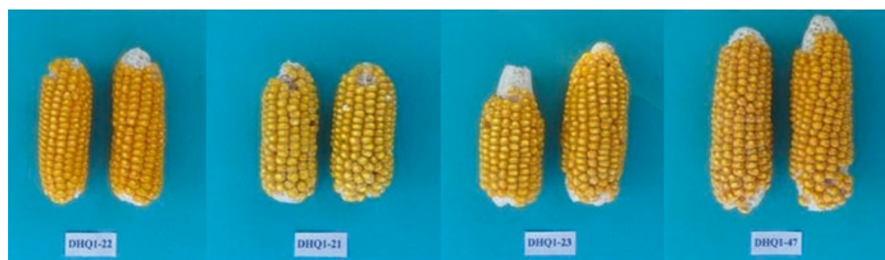

Fig. 12.3 Mature cobs of QPM doubled haploid (DH) lines derived from Vivek QPM 9

12.4 Gene/Genome Editing

Maize is a popular crop for conducting genetic studies. All available genome editing tools—meganucleases, zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/CRISPR-associated nuclease protein (Cas) system—have been used for targeted modification of genes. Gao et al. (2010) used meganucleases for editing LG1 (liguleless) gene in maize, and Djukanovic et al. (2013) developed male-sterile transgenic plants using redesigned I-CreI homing endonuclease to introduce mutations in the fifth exon of the cytochrome P450-like maize male fertility gene MS26. Zinc-finger nucleases (ZFNs) have been used for herbicide resistance and phytate-reduced maize (Shukla et al. 2009) and stacking of herbicide resistance genes (Ainley et al. 2013), while transcription activator-like effector nucleases (TALENs) have been used for developing maize with reduced epicuticular wax in leaves (Char et al. 2015) and induction of haploid plants (Kelliher et al. 2017).

However, it is the CRISPR/Cas systems that on account of their ease of use combined with high precision are increasingly becoming the preferred choice of researchers for carrying out gene-editing work. CRISPR/Cas9 system comprises a Cas9 endonuclease of *Streptococcus pyogenes* origin and single-guide RNA (sgRNA) which is a synthetic RNA of about 100 nucleotide length. Its 5′-end has a 20-nucleotide sequence that guides Cas9 to a target DNA sequence in the genome. Cas9 effects a double-strand break at the target site. The repair is performed by nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) DNA repair mechanisms. When supplied with an exogenous oligo template, HDR induces specific sequence alterations. CRISPR/Cas system has been used for developing maize with high amylopectin, thermosensitive male sterile lines, herbicide resistance, and drought tolerance.

Constitutive overexpression of ARGOS8 driven by Ubiquitin 1 in the transgenic plants increases grain yield under drought stress conditions without yield penalty in nonstress environments (Shi et al. 2015). Shi et al. (2017) used CRISPR/Cas to

generate novel variants of ARGOS8 gene with moderate level of constitutive expression of the gene. Two genome-edited variants *ARGOS8-v1* and *ARGOS8-v2* showed significantly higher expression of ARGOS8 mRNA than that in wild-type plants. In these variants, ARGOS8 protein was detectable in developing kernels also but was absent in the wild type. Under field evaluation, the yield of the ARGOS8 variants was 0.33 t ha^{-1} more than the wild type under stress at flowering stage. Under well-watered conditions, the variants showed no loss of yield. Svitashev et al. (2015) targeted five different genomic regions: male fertility genes (Ms26 and Ms45), upstream of the liguleless1 (LIG1) gene, and acetolactate synthase (ALS) genes (ALS1 and ALS2). Mutations were subsequently identified at all targeted sites, and plants carrying biallelic multiplex mutations at LIG1, Ms26, and Ms45 were retrieved. Editing the ALS2 yielded chlorsulfuron-resistant plants. Mutation of the ZmTMS5 gene using the CRISPR/Cas9 technology has generated maize tms5 male-sterile mutants that are thermosensitive. Chen et al. (2018) targeted male sterility 8 (MS8) and generated novel ms8 mutant lines using the CRISPR/Cas9 system. The two types of mutations that occurred had an adenine nucleotide insertion (ms8-InA ms8-DelG) and a guanine nucleotide deletion (ms8-DelG) and resulted in frameshift and a nonfunctional MS8 protein. The male-sterile phenotype could be stably inherited by the next generation in a Mendelian fashion. Transgene-free ms8 male-sterile plants were obtained by screening the F_2 generation of male-sterile plants.

Kelliher et al. (2019) developed a novel method, named HI-Edit, which enables the direct genomic modification of commercial varieties. A native haploid inducer line was used for testing the HI-Edit in sweet corn and can be extended to dicots through an engineered CENH3 HI system. They also recovered edited wheat embryos by using Cas9 delivered through maize pollens. They unveiled that a transient hybrid state foregoes uniparental chromosome elimination in maize HI. The edited haploid plant lacks both the editing machinery and haploid-inducer parental DNA. The Haploid-Inducer Mediated Genome Editing (IMGE) approach developed by Wang et al. (2019) that integrates DH and CRISPR/Cas9 technologies is possibly the shortest method to achieve completely homozygous lines with the desired target trait. The approach utilizes a maize haploid inducer line carrying a CRISPR/Cas9 cassette targeting for a desired agronomic trait to pollinate an elite maize inbred line and to generate genome-edited haploids in the elite maize background. The gene targeted to demonstrate the approach was ZmLG1, the loss of function versions of which produce genotypes that lack auricle and ligules and are referred to as liguleless1. They used CAULG1-Cas9 (haploid inducer genotype carrying ZmLG1 CRISPR/Cas9 cassette) to pollinate B73. Ten out of 245 resultant maternal haploid plants exhibited lg1 mutant phenotype, giving editing efficiency of about 4.1%. Screening for Basta resistance and genotyping with the CRISPR/Cas9 cassette-specific marker S1 confirmed that all the ten lg1 maternal haploids were Cas9-free. Spontaneous chromosome doubling in three of these ten liguleless plants yielded DH seeds. Using this method, homozygous DH lines with the desired trait could be delivered in two generations as against six to seven generations typically required to introgress a target trait into elite breeding lines.

12.5 Conclusion

Being an important source of energy and nutrition for a large section of global population, biofortification of maize has enormous implications for global food and nutritional security. Biofortified maize constitutes a relatively inexpensive means of delivery of essential nutrients to malnourishment-prone regions of the world by being more easily accessible compared to other alternatives for malnutrition alleviation. Development of biofortified maize has gained momentum in the last two decades, and maize genotypes rich in lysine, tryptophan, provitamin A, iron, and zinc and low in phytate have been developed in shorter periods of time through the use of molecular breeding tools, mainly marker-assisted selection. While availability of robust molecular markers has led to appreciable progress in developing maize varieties rich in lysine, tryptophan, and provitamin A, lack of the same for other nutritionally important components such as methionine, iron, and zinc has impeded biofortification of maize for these traits. Application of doubled haploid technology holds promise for quicker fixation of such traits with low heritability owing to high environmental sensitivity. Transgenic approaches and targeted gene editing have proved effective in creating novel sources for many nutrition-related traits that augment the variability for these traits. Integration of molecular markers with DH technology and gene editing offers vast opportunity for further reduction in product delivery time with enhanced precision.

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Chapter 13
Efficient Barley Breeding

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13.1 Introduction

Barley (*Hordeum vulgare* L.) is a member of the grass family. It is a self-pollinating, diploid species with 14 chromosomes. It is an important cereal crop in the world ranking next to maize, wheat, and rice. It is one of the earliest domesticated food crops since the start of civilization. Barley (*Hordeum vulgare* L.) is grown over diverse eco-geographical environmental conditions as compared to other crop species because of its hardiness to environmental variations. Barley is often considered the only possible rainfed cereal crop under low input and stressful environments, like drought, heat, and cold. This adaptability to extreme and marginal conditions has led to widespread cultivation of this cereal throughout the world. The range of barley cultivation is from the tropics to high latitudes (>60° N) in Iceland and Scandinavia as well as in high latitudes up to 4500 meters above sea level (masl) in the Himalayas (von Bothmer et al. 2003; Ceccarelli et al. 2008). Historically, owing to its rich dietary fiber and readily available energy, barley was utilized by the Roman gladiators, who were also called as "hordearii" (Andrew 2008). Although globally the major utilization of barley is for feed and malting purposes, because of its nutritional value, barley is consumed as a staple food in North and Sub-Saharan Africa (SSA), Central Asia, and South-West Asia. In India, barley is an important cereal in winter after wheat in both area and production. Due to its very hardy nature, barley is successfully cultivated in adverse agro-environments like drought,

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salinity, alkalinity, etc., in varied topographical conditions like plains and hilly areas under rainfed and irrigated conditions. Barley is generally considered as a poor man's crop because of its input requirement and better adaptability to harsh environments. Area under this crop is mainly concentrated in the states of U.P., Punjab, Rajasthan, Haryana, M.P., and Bihar and also in the plains of H.P., Uttaranchal, and J&K.

Barley is botanically known as *Hordeum vulgare* and is believed to originate in western Asia or Ethiopia during the Stone Age, and in accordance with the archaeological evidence, the earliest signs of barley cultivation date back to Neolithic times. Several findings from around 7000 BC show that barley was one of the first crops under domestication. The original area of cultivation was the Fertile Crescent in the Near East, from present-day Israel and Jordan via Syria and southern Anatolia to the Zagros Mountain area in western Iran.

One of the first proposals for the evolution of cultivated barley was that of de Candolle in 1895, and it has been much debated since that time. The two possibilities that de Candolle considered were (1) that two-rowed barley had given rise to four- and six-rowed barley in prehistoric times or (2) that the ancestors of four- and six-rowed barley had become extinct. The views on the origin have shifted during intervening years, depending on the opinion of whether barley comprised several species (*H. vulgare*, *H. distichum*, *H. irregulare*, *H. deficiens*, *H. agriocrithon*, *H. spontaneum*, etc.) or whether it was to be regarded as one species only. The main points of reasoning have been based on morphology of the spike: whether it was two, four, or six rowed. Nevski in 1941 concluded that barleys are of a diphyletic origin; that is, the progenitor of both two- and six-rowed barley had more developed lateral spikelets than *H. spontaneum*, together with a fragile rachis. From this progenitor, the six-rowed and later two-rowed barleys were derived, both obtaining the tough rachis during domestication. He considered that the lateral spikelets *of H. spontaneum* were much reduced to be able to evolve into forms with the large, fertile lateral spikelets of six-rowed barley. The works of Aberg and Wiere (1948) in Tibet proclaimed that the ancestral, six-rowed, brittle-rachis barley had been found: *H. agriocrithon*. He suggested several ways in which the cultivated six- and two-rowed forms could have evolved, all originating from Central Asia and later spreading westward, where they came into contact with *H. spontaneum*.

One of the main arguments in the debate over the evolution of cultivated barley is the genetics behind the brittle versus tough rachis is controlled by two genes, the cultivated barleys being homozygous recessive for either one or both. The genetics of two- versus six-rowed spike forms has proved to be somewhat more complicated. It involves at least two loci on two different chromosomes, each of them being represented by multiple allelic series.

In conclusion, there are several points which remain in doubt concerning which pathways the domestication of barley has followed. But all indications point to *spontaneum* as the most probable immediate ancestor (among known forms) of cultivated barley, and all the four- and six-rowed forms that occur in cultivation and as weeds in the fields are the results of accumulated mutations and hybridization. The

ancestor of *spontaneum* was no doubt a form with more or less developed lateral spikelets, perhaps male-fertile like *H. murinum* and *H. bulbosum.*

13.2 Barley Production Worldwide

Barley production traditionally has been important in the world. The total area harvested each year is around 50–80 Mha and ranked 4th after wheat (~200 Mha), rice (120–150 Mha), and corn (100–150 Mha) (reference). In the recent two decades, the area has been declining from more than 80 Mha to around 55 Mha. Among the major barley production countries (Table 13.1), Russia (including all the countries from former USSR), the United States, India, and China are the major countries showing the significant decrease in barley cropping area. The production area in USSR has decreased from around 30 Mha in the 1980s to around 17 Mha recently with Russia being one of the major countries showing significant decrease (from more than 16 Mha in the 1980s to around 10 Mha in the 2000s). In United States, the production area has reduced from around 5 Mha in the 1960s to around 1.4 Mha recently. Barley was replaced by commercially more attractive corn and feed wheat in the feed mix. The Chinese barley production area has reduced from more than 5 Mha in the 1960s to less than 0.8 Mha today. India showed a similar trend with China, from more than 3 Mha to less than 0.8 Mha, but the decrease in the barley production area is mainly due to the significant increase in wheat production area (from 13 Mha to 28 Mha) while the Chinese wheat production area has been relatively consistent or even reduced slightly. In contrast to above major barley production countries, Australia showed significant increase in production area, from 1 Mha in the 1960s to 4 Mha in the 2000s, mainly due to the total increased cropping area (Statistics from United States Department of Agriculture).

13.3 Domestication and Cultivation of Barley

The original area of barley cultivation is assumed to have been the Fertile Crescent, stretching from present-day Israel and Jordan to Syria and Southern Anatolia and to the Zagros Mountain area in Western Iran (von Bothmer and Jacobsen 1985). However, with evidence found in Morocco (Molina-Cano and Conde 1980; Moralejo et al. 1994; Molina-Cano et al. 1987, 1999, 2002) and in southeastern Himalaya and Tibet (Xu 1982), the general agreement on the mono-centric origin of barley was challenged. Still the Near East, Tibet, Abyssinia, and the Western Mediterranean region were proposed as centers of diversity for barley. The Abyssinian region in the eastern part of *Africa*, including the modern countries as Ethiopia, Eritrea, Djibouti, and Somalia, is believed to represent an important center of diversity for barley. Barley is believed to have been cultivated in this region as early as 3000 BC (Gamst 1969). The region possesses diversity of climates, soils, topography, social

Table 13.1 Top countries for barley area (ha), yield (hg/ha), and production (tonnes)

Country	Area harvested (ha)	Country	Yield (hg/ha)	Country	Production (tonnes)
Russian Federation	7,847,738	Ireland	83,563	Russian Federation	20,598,807
Australia	4,834,102	Germany	69,302	Australia	13,505,990
Spain	2597527	France	63115	Germany	10,853,400
Ukraine	2501500	United Kingdom	60909	France	10,545,427
Turkey	2,418,312	Denmark	59,998	Ukraine	8,284,890
Canada	2,197,632	Austria	56,300	Canada	7,891,300
Kazakhstan	2,068,766	Sweden	52,850	United Kingdom	7,169,000
Morocco	2,001,473	Hungary	52,734	Turkey	7,100,000
France	1,670,831	Croatia	48,272	Spain	5,785,944
Iran (Islamic Republic of)	1,600,000	Bulgaria	46,371	Denmark	3,992,300
Germany	1,566,100	Slovakia	45,316	Poland	3,793,032
Algeria	1,303,131	Argentina	42,991	Argentina	3,741,158
United Kingdom	1,177,000	Finland	40,751	Kazakhstan	3,305,224
Ethiopia	955,029	China	40,583	Iran (Islamic Republic of)	3,100,000
Poland	953,784	Poland	39,768	United States of America	3,090,010
Argentina	870,213	Italy	39,289	Morocco	2,466,462
Syrian Arab Republic	804,124	United States of America	39,076	Ethiopia	2,031,661
United States of America	790,760	Canada	35,908	China	1,897,318
Denmark	665,400	Ukraine	33,120	India	1,750,000
India	656,000	Turkey	29,359	Sweden	1,635,200
China	467,515	Australia	27,939	Ireland	1,505,800
Finland	358,300	India	26,677	Finland	1,460,100
Sweden	309,405	Russian Federation	26,248	Hungary	1,404,356
Hungary	266,308	Brazil	24,664	Italy	984,281
Italy	250,526	Spain	22,275	Algeria	969,696
Iraq	197,625	Ethiopia	21,273	Austria	782,029
Ireland	180,200	Iran (Islamic Republic of)	19,375	Syrian Arab Republic	780,000
Austria	138,903	Kazakhstan	15,977	Bulgaria	595,237
Bulgaria	128,365	Iraq	15,338	Slovakia	545,285
Brazil	122,019	Morocco	12,323	Iraq	303,114
Slovakia	120,329	Syrian Arab Republic	9700	Brazil	300,947
Croatia	53,950	Algeria	7441	Croatia	260,426

environments, vegetation cover, and livestock. The first "Ethiopians" to have cultivated barley are believed to be the Agew people, in about 3000 BC. Evidence from a flavonoid study also raised doubt on a mono-phyletic origin of barley, arguing that through long-term introgression of the relatively fewer wild relative genes remained swallowed in the gene pool of cultivated barley in Ethiopia (Endeshaw 1983). Furthermore, very recent work considered Ethiopia an independent center of barley diversification and a potential domestication site (Orabi et al. 2007). Indeed, Ethiopian barleys have been isolated so long that two of them, irregular barley and deficient barley, were for a time considered distinct species. The long history of barley cultivation and the diversity in soils, climate, altitude, and topography together with geographical isolation for long periods have resulted in a large number of landraces and traditional agricultural practices (Berhanu et al. 2005). There is high diversity observed among Ethiopian barleys with six-rowed, two-rowed, deficiens, and irregular types, hulled and hull-less, and with different grain colors. The variation in Ethiopian barleys has been attributed to mixing of types in the fields and associated with mutation, frequent natural hybridization, and disruptive selection (Zemede 1989). The variation might have resulted from the highly heterogeneous environment, on which farmers' preferences and selection for desirable traits are imposed. Recent population genomics studies toward understanding intriguing patterns in the cultivated barley genomes that shed light on the trajectory of barley domestication have postulated that there are three possibilities for the domestication/origin of cultivated barley. The first is that the hypothetical wild progenitor population could have had a highly admixed ancestry that was passed down to the cultivated lineage. The admixed wild barley genotypes frequently occur at the contact zones between the modern wild populations. The second hypothesis is that the wild progenitor lineage was not admixed and the recurrent gene flow from wild into the proto-domesticated populations happened during the transition to cultivation gradually creating the heterogeneous admixture patterns. The third and perhaps the likeliest scenario is a combination of the ancestral population structure and the gene flow (Pankin and von Korff 2017). Important mutations occurred, such as adaptation to different climatic and edaphic conditions, during an early stage of cultivation which promoted the migration process of barley into other parts of the world. Few important characters which were inherited during the process of barley domestication are discussed below.

13.3.1 Brittleness of Rachis

Shattering is a character of natural adaptation in wild plants. The brittleness of rachis in barley promotes the spreading of seeds together with the rough awn, which may easily attach to animals for effective dispersal. The detached rachis segment with one spikelet in ssp. *spontaneum* (one dispersal unit) is shaped like an arrowhead which is why it is difficult to pull the seed out after it attaches somewhere. One of the most important traits for the domestication of barley is probably non-brittleness

of rachis which is of benefit for an efficient harvest without loss of grains. In archaeological remains, wild forms of barley with fragile spikes were found in some quantity. The elimination of such a deleterious character was probably the first requirement for a cultivated form.

13.3.2 Kernel Row Type

There is a wide variation in kernel row type in barley. The row type is basically controlled by the gene *vrs1*, and six rowed is recessive to two rowed. Several genes showing imperfect six rowed, such as *vrs2* or *vrs3*, have been found mainly in artificially induced mutants. The development of lateral kernels is also controlled by the gene *int-c* (*intermedium* spike-c), which regulates the size of lateral spikelets.

13.3.3 Covered and Naked Kernels

Naked kernel is a single recessive character from the covered wild type. Naked barley is distributed widely in the world, but there is a higher preference for naked barleys in East Asian countries such as China, Korea, and Japan, and it is especially high in Tibet and the northern parts of Nepal, India, and Pakistan. Since the frequency is low in the west, Vavilov (1926) considered southeastern Asia to be a center of origin for naked barley. In these areas, barley is used as a major human diet and naked barley is preferred.

13.3.4 Dormancy

Dormancy is a natural adaptational system controlling the seed germination in semiarid areas where barley was domesticated. Most of the wild accessions of ssp. *spontaneum* are highly dormant. Compared to other characters which might have been important for the domestication, the genetic system of dormancy seems more complicated. A certain level of dormancy is also useful in cultivated material to prevent preharvest sprouting or unnecessary starch degradation during the storage period.

13.3.5 Growth Habit

One of the prerequisites for expansion of the cultivation area for barley must have been differentiation of spring habit. In high latitudes and in mountainous regions, barley is sown in spring to avoid damage by a severely cold winter. Accordingly, in

these regions, spring type cultivars are needed in order to grow and head normally. At low latitudes, on the other hand, air temperature is too high to induce vernalization in a winter type. Spring type cultivars prevail in these regions. The genes *sgh1*, *Sgh2*, and *Sgh3* are all regulating spring habit, and their allelic genes winter habit. Because of the epistatic effect among these genes, only a single genotype, *Sgh1sgh2sgh3*, exhibits winter habit. Almost all strains of *Hordeum vulgare* ssp. *spontaneum* are of winter habit. Consequently, the first barley types to be domesticated might have been of winter habit type, but a dominant mutation occurred first in the *sgh2* locus, resulting in spring barley.

13.3.6 Productivity and Quality Traits

Wide cultivation of barley results in increased probabilities for natural mutational events. Direct or indirect selections in cultivation have increased the ratio of genotypes preferred by humans. Productivity is apparently the largest concern for human consumption, but it is a complex character consisting of physiological changes, adaptations of the genotype, reductions of yield losses, etc. Human needs for quality characters such as for malting purposes or human diets resulted in strong selections on the barley populations. Once categories such as malting varieties or naked semi-dwarf food barley were established, introductions of other sources of variation were restricted. In this way, some of the promising sources of variation from the wild or primitive germplasm during the process of domestication and migration might have been lost. For example, some genes with high level of enzymatic activities, of potential importance for malting, are present in ssp. *spontaneum* collections as reported, but not in cultivated forms.

13.3.7 Disease Resistance

A clear host-pathogen co-evolution system has been suggested in barley fungal diseases, especially in mildew resistance. Even in wild *Hordeum* species, mildew resistance is a key to estimate genetic distance among species. The diversity of disease resistance in ssp. *spontaneum* is wide compared to cultivated barley. Primitive barleys or landraces have basically a fairly high level of resistance to most diseases, and bottlenecks might differentiate the resistance of germplasm. For example, Barley Yellow Mosaic Virus (BaYMV) was a local barley disease in Japan until recently and has spread into other countries such as China and Germany. But a high level of resistance to disease is found in Ethiopian germplasm. Differences between germplasm can also be found in the reaction to *Pyrenophora teres* f. *teres* in Nepalese barleys. Naked food barley, distributed in the Himalayan region, has a high level of resistance to the Canadian isolate WRS102, but hulled barley, distributed mainly in the Indian subcontinent, is quite susceptible to the isolate.

13.3.8 Abiotic Stress Tolerance

A major reason for the increase of barley cultivation during the Mesopotamian age might have been higher tolerance to salt stress in barley than in wheat. When salinity began to increase in the irrigated land of southern Mesopotamia, wheat production declined, and a near monoculture of barley was established. There is no evident geographical differentiation for salt tolerance at the seedling stage, and most wild accessions showed better tolerance to salinity than cultivated material. Some genotypes of ssp. *spontaneum* have the ability to survive at a very high level of salinity percentage. Tolerance to excessive moisture is one of the key adaptive traits for cultivation in an Asian monsoon climate, especially when grown as a winter crop in paddy fields. In barley, this type of tolerance is expressed during germination and early developmental stages. And most of the East Asian accessions are tolerant to this type of stress.

13.4 Breeding Goals

Barley productivity throughout the world is affected due to various biotic stresses like rust, powdery mildew, net blotch, leaf blight, etc., and abiotic stresses like drought, submergence, and salinity. Exposure to these stress conditions induces disruption in plant metabolism leading to substantial decrease in fitness and productivity of a crop. With the prevailing diversity of the causal pathogens, concurrent evolution of new virulent strains, and changing climatic conditions, the management of the biotic and abiotic stresses is becoming ever challenging. The most effective and sustainable way of management of these stresses is the enhancement of host resistance which is an economical and environmental friendly approach. Therefore, knowledge about a better understanding of the barley genome, breeding methods, and cultivation technologies is of vital importance. Also, identification of superior genotypes for disease resistance through marker-assisted selection (MAS) and pyramiding of multiple disease resistance genes into barley varieties may contribute to greater longevity of disease resistance. Here, we review the genetic diversity studies, breeding work, and role of molecular approaches for the improvement of barley vis-à-vis different biotic factors. Work of different researchers against various biotic stresses highlighted can be utilized by breeders for the generation of new improved varieties of barley.

13.4.1 Barley for Feed and Food

Barley is a multipurpose cereal crop grown for food, malting, and general purposes (feed) throughout the world. Whereas relatively lesser amount of barley is utilized precisely for food nowadays, it has incredible prospective to regain some of its importance as a food grain, mainly because of its high nutritive esteem. Barley grain offers lesser fat complex sugars, primarily starch for vitality; comparatively well-

adjusted protein content to meet amino acid necessities; vitamins, mainly vitamin E; minerals and various other antioxidants, mainly polyphenolics; and soluble and insoluble fiber with general (quick passage of food through colon) and definite health benefits. Barley is the main cereal used in the production of malt throughout the world because it contains numerous enzymes which are essential for transforming the grain starches into several types of sugars like glucose (monosaccharide), maltose (disaccharide), maltotriose (trisaccharide), and the complex sugars known as maltodextrins. It also contains additional enzymes, for instance, proteases, which cause the protein breakdown in the grain into different forms that can be exploited by yeast. Therefore, malted barley grain is used in the production of beer, malted milkshakes, malt vinegar, whisky, confections like maltesers and whoppers, flavored drinks like Horlicks, Milo, and Ovaltine, and some baked goods. For the ruminants, barley is the third most promptly degradable cereal after wheat and oats. So there are various potential opportunities of barley like malting, food, and feed purposes, and the potential is great to enhance barley for all these practices.

13.4.1.1 Malting/Brewing

About 10% of worldwide production of barley is utilized to make malt for preparing lager (Fig. 13.1). The malting varieties incorporate two-rowed, six-rowed, hulled, and hull-less varieties, yet the hulled barley is favored because hull adds flavor and

Fig. 13.1 Micro malting system for extraction of malt form barley genotypes

helps in filtration during the process of brewing (Gunkel et al. 2002). Malting barley varieties are for the most part produced for a particular market, e.g., for trade or domestic brewing. The chemical, biochemical, and physical properties of barley grain can largely affect the malting procedure and quality of beer. Kernel physical characteristics like germ growth, kernel maturity, germination percentage, size, frost damage, and measure of seed-borne maladies are factors that influence malting process. The measure of grain protein, β-glucan, and starch and their interactions in the period of grain filling influence grain hardness and yield of malt extract (Psota et al. 2007). Another factor that defines the quantity of malt extract is the level of alpha-amylase. Soft barley varieties are usually preferred for malting (Gupta et al. 2010) with protein content ranging from 10.5% to 13.0% for six-rowed varieties and 10.5% to 12.5% for two-rowed types. Barley cultivars having higher protein concentration (>15%) are not used for malting because they need longer steeping time, produce low malt extracts, and have erratic germination (Swanston and Molina-Cano 2001). Bleached barley grain is additionally not suitable for malting because during the breakdown of phenolics, the production of undesirable flavors takes place in lager (Mussatto et al. 2006). An effective maintenance of malting barley trade market wants great determination of cultivars with suitable malting qualities.

13.4.1.2 Livestock Feed

Barley is regularly utilized for animal feed in spite of the fact that nutritive value is lesser as compared to wheat or corn. Both six- and two-rowed hull-less barleys are usually grown for the production of animal feed. The removal of the hull is required to result in uncertain increments in absolute nutrient levels of hull-less barley when compared with regular barley. In practice, the nutrient composition of hull-less barley often exceeds this expectation. That is why the interest is growing toward the development of hull-less barley for the feed industry. Moreover, a substantial measure of hulled malting barley grain with inadequate malting characteristics entered into the animal feed market. This makes feed barley a nonhomogeneous entity with changing nutritive esteem. The six- and two-rowed hull-less barleys produced for feed in Canada are moderately higher in protein content (14–15%); however, the two-rowed barley grain is favored because it has a relatively higher content of carbohydrates (Fregeau-Reid et al. 2001) and is highly edible by monogastric animals like swine and poultry.

When the monogastric animals are fed with hulled barley, they will not be able to digest the fibrous hull; therefore, to help in digestion, an enzyme named β-glucanase is often supplemented to the diet of these animals (Mathlouthi et al. 2003). Other than hulls, an additional component of seed is phytate that adversely influences barley consumption as animal feed. Phytic acid effectively chelates polyvalent cations like copper, iron, calcium, magnesium, zinc, and aluminum, therefore making the minerals inaccessible for absorption (Adams et al. 2002). Zinc is one of the most vulnerable minerals to chelation by phytic acid. A decrease of phytic acid

formation in barley genotype HB379 (Roslinsky et al. 2007) has multiplied the accessibility of zinc and phosphorus for broilers (Linares et al. 2007). Despite the fact that barley is prevalent as animal feed, barley grain possessing higher concentration of starch is not acceptable for ruminants. Quick fermentation of starch in rumen causes drop in pH, which ultimately decreases fiber consumption and causes various gastric disorders. Decrease in milk fat content is found when barley with high starch concentration is utilized as feed for lactating bovines (Larsen et al. 2009). For that reason, it is essential to examine seed constitution while choosing barley grain for ruminants as well as non-ruminants.

13.4.1.3 Food

Commonness of lifestyle diseases is increasing step by step. For the most part, the youthful age does not have much responsiveness about healthy nourishing supplements. Barley is a cereal grain that is rich in nutritive components with so numerous health benefits like weight loss; declining blood pressure, blood glucose, and blood cholesterol; and prevention from colon cancer. It is an effectively accessible and modest grain, and it contains both insoluble and soluble fiber; protein; vitamins B and E; minerals like magnesium, iron, and selenium; anthocyanins; and flavonoids. Therefore, hull-less barley is preferred nowadays in foods.

13.4.1.4 Cholesterol-Free or Lower in Fat Content

Because of the presence of a lesser amount of cholesterol in hull-less barley, doctors recommend to eat barley food to the heart patients. Although barley is cholesterol-free, it also possess cholesterol lowering property and hence creates hypocholesterolemic effects inside the body. Various studies have demonstrated that soluble fiber β-glucan from barley and oat can bring down the low-density lipoprotein (LDL) and total cholesterol level and therefore plays an important role in both the anticipation and management of cardiovascular ailment. The FDA established that everyday utilization of 3 g of soluble dietary fiber β-glucan from barley or specific dry milled barley would create a similar cholesterol-bringing down impact as oat products (decreasing total plasma cholesterol level by 5–8%).

13.4.1.5 Vitamins and Minerals

Barley is also a principal source of various vitamins and minerals like niacin, thiamine, selenium, iron, magnesium, zinc, phosphorus, and copper. The mineral content of barley kernel varies from 2% to 3%, depending on the genotype. The minerals present in the seed are mainly found in the aleurone, embryo, as well as in pericarp tissues (Marconi et al. 2000). Minerals which influence the kernel nutritional value are mainly divided into macro- and micro-elements on the basis of their concentra-

tion in foods. The macro-elements comprise calcium, magnesium, phosphorus, and potassium. The others are sulfur, silicon, and chloride. The nutritionally essential micro-elements present in the barley grain are zinc, selenium, iron, cobalt, copper, and manganese. Phosphorus and potassium are the macro-elements which are most plentiful in terms of availability and nutritive qualities. Phosphorus present in barley kernel occurs in the form of phytic acid. The enzyme phytase is absent in monogastric animals for the consumption of phytic acid. The higher content of phytic acid chelates various monovalent ions like copper, zinc, and calcium making them inaccessible. Comparatively lesser phytic acids are required for poultry feed as higher content causes sticky droppings.

Zinc helps in healing injuries and also works wonders for the skin, whereas selenium plays a major role in reducing the risk of colon cancer. Selenium additionally plays an important role in various metabolic pathways: antioxidant defense systems, immune function, and thyroid hormone metabolism. Selenium has been shown to initiate DNA repair and amalgamation in damaged cells, to hinder the cancer cell proliferation, and to prompt their *apoptosis*, which is the self-destruct sequence the body uses to remove worn-out or abnormal cells. Copper is another trace element provided by barley, which is helpful in reducing the symptoms of rheumatoid arthritis. Phosphorus plays a vital role in forming the mineral matrix of bone and also helps in the formation of several life critical compounds comprising *adenosine triphosphate* (ATP: which is the energy currency of body). Phosphorus is the main component of nucleic acids which are the building blocks of genetic code. Copper is an important cofactor of crucial oxidative enzyme named superoxide dismutase (SOD). SOD deactivates free radicals produced in mitochondria.

13.4.1.6 Antioxidants and Phytochemicals

Phenolics are one of the major classes of phytonutrients, and they are potent antioxidants that work in numerous ways to prevent diseases. Various compounds are incorporated in this category, for example, ellagic acid, curcumin, catechins, quercetin, and many more. Barley grains comprise a varied range of phenolic acids that are either cinnamic acid or benzoic acid derivatives. In general, larger amounts of phenolic compounds were accounted for oat and barley in contrast with rye and wheat (Zielinski and Kozlowska 2000). The phenolic acid which is present in large amounts in cereals is ferulic acid, signifying up to 90% of overall polyphenols (Sosulski et al. 1982). Similarly, Naczk and Shahidi (2006) and Hernanz et al. (2001) stated that ferulic acid is the principal phenolic acid present in barley grain. Yu et al. (2002) examined the phenolic acid composition in 30 barley cultivars where they establish changing levels of cinnamic and benzoic acids. Phenolic compounds are thought to exhibit several roles like reducing agent, free radical scavenger, potential producer of pro-oxidant metals, and it prevents the formation of singlet oxygen.

Anthocyanins present in barley and wheat are discovered either in the aleurone layer or the pericarp and bring about blue and purple hues of grain color. The black

coloration of the pericarp and lemma of barley is depicted because of the presence of melanin-like pigment in them (Lundqvist et al. 1996) which might overlap additional pigments. The black color of the kernel because of melanin-like pigment is unidentified to wheat cultivars.

Zeaxanthin and lutein are the two fundamental carotenoids recognized in barley (Panfili et al. 2004). They effectively function as free radical scavengers because of the presence of electron rich chain (Cooke et al. 2002) and hinder the free radical propagation reactions, for example, lipid peroxidation. Zeaxanthin and lutein are accountable for the pigmentation of the macula lutea present in the retina, which is the area of best visual sharpness. Therefore, dietary zeaxanthin and lutein are thought to provide protection against cataract and age-related macular degeneration (Beatty et al. 2000). Moreover, zeaxanthin and lutein probably act together with various other bioactive components against cardiovascular risk, cancer, and different infections (Mares-Perlman et al. 2002; Calvo 2005). Tocopherols or vitamin E are additionally present in noticeable amounts (Cavallero et al. 2004; Andersson et al. 2008). Another kind of phytonutrient rich in barley is plant lignans, which are converted by friendly flora present in digestion tracts into mammalian lignans, comprising one called enterolactone that is supposed to provide protection against breast and various hormone-dependent cancers along with heart disease.

13.4.2 Malt Barley Improvement

Earlier, barley was considered as high energy food as it comprises eight vital amino acids and many potential antioxidants. Takahashi (1955) indicated by an examination that hull-less barley is extensively dispersed; however, its frequency significantly varied among locales. The distribution of hull-less barley is skewed in the direction of East Asia as 95% of it grows in the plateaus of Japan, Korea, Nepal, Tibet of China, and Bhutan. Hull-less barley is comparatively grown in Ethiopia at low recurrence (Assefa and Labuschagne 2004) and has been not really grown in Australia along with the western world. Hull-less barley hereditary assets in China are richest throughout the world, and it possesses 77% of the world's aggregate hull-less barley hereditary assets (Lu 1995). Barley cultivars vary anatomically in the spike structure, i.e., two- and six-rowed cultivars, or in the growth pattern like between spring- and winter-type barley genotypes. Hulled barley has the caryopsis covered with the hull. Hull-less barley is rich in nutritional constituents like β-glucan, limiting amino acids, starch, and total dietary fiber as compared to its hulled types (Boros et al. 1996). Hull-less barley has greater levels of starch and lesser fiber concentration; therefore, it has greater digestible energy (Shon et al. 2007). There is a noteworthy rise in the production of hull-less barley for the diets of non-ruminant animals who are not able to digest the fibrous hulls of the hulled barley and for food thickeners, health foods, and breakfast cereals for human consumption because it has several nutritional characteristics (Edney et al. 1992). The two different forms of hull-less barley are waxy and normal. The amylose to amy-

lopectin ratio in the normal form of hull-less barley is similar to the regular hulled barley (around 25% amylose and 75% amylopectin). The waxy form of hull-less barley has higher content of amylopectin, starch, and β-glucans (about 95–100% amylopectin). Because of higher β-glucan content, waxy barley is not easy to digest which makes it highly suitable for its use as thickening agents in various industries. Regular hull-less barley cultivars possess higher β-glucan content as compared to hulled types. The β-glucan inhibits rise in the LDL cholesterol levels in humans. Barley types differ considerably in starch, β-glucan, and grain test weight but not in the protein content, with hull-less barley possessing significantly greater grain test weight and starch content as compared to hulled and malting barleys and significantly greater β-glucan content as compared to malting barleys (Griffey et al. 2010). Barley was probably used in food but progressed mainly into feed and malting grain due to the increase in eminence of rice and wheat. At present, 94% of globally produced barley is used for the manufacturing of beer, and only 2% is used in food. On the other hand, it is essential for human nourishment in the areas where hull-less barley is developed at much higher frequencies. As of late, it is pulling in significant consideration as a nutritious human sustenance in nonconventional areas because of its higher content of β-glucan, which goes about as inhibitor in the synthesis of cholesterol (Berglung et al. 1993), and its higher lysine (Bhatty 1986) and protein content (Oscarsson et al. 1996) and owing to processing benefits without the removal of hull for the barley food trade (Newman 1992; Bhatty 1993). There are likewise ongoing increments in the utilization of naked barley in western nations. Therefore, hull-less barley is now used in various kinds of food products, and their consumption provides potential health benefits.

13.4.3 Breeding Barley for Abiotic Stress Tolerance

Abiotic stress factors such as heat, cold, drought, salinity, and nutrient stress have a huge impact on world agriculture, and it has been suggested that they reduce average yields by >50% for most major crop plants (Bray et al. 2000; Wang et al. 2003). It is widely accepted that the most characteristic and general response of plant cells under various stresses is an increase in reactive oxygen species (ROS) production. Due to their signal molecule function, ROS plays a crucial role in the signalization of adverse conditions for cells and in the activation of defense responses. However, during more severe and persistent stress conditions, an uncontrolled accumulation of ROS may occur, which causes several damages including membrane and protein modifications in cells, enhanced level of lipid and protein peroxidation, and activation of antioxidant apparatus. Plants are equipped with both non-enzymatic antioxidants and enzymatic ROS scavengers to protect cells from oxidative damage (Breusegem et al. 2001). Abiotic stresses resulting from excessive salinity or water deficit led to reduction in photosynthesis, transpiration, and other biochemical processes associated with plant growth, development, and crop productivity (Shannon 1997; Tiwari et al. 2010). Furthermore, abiotic stress leads to oxidative stress in the

plant cell resulting in a higher leakage of electrons toward O_2 during photosynthetic and respiratory processes, leading to enhancement of reactive oxygen species (ROS) generation (Asada 2006). Much of the injury on plants under abiotic stress is linked to oxidative damage at the cellular level leading to cell death (Mittler 2002). During optimal growth conditions, balance between ROS formation and consumption is tightly controlled by plant antioxidant defense system (Hameed et al. 2011). Plants containing high activities of antioxidant enzymes have shown considerable resistance to oxidative damage caused by ROS (Gapinska et al. 2008). Barley (*Hordeum vulgare* L.) is one of the most important small cereal grain crops and commonly used in malting and brewing industry for animal feed and human consumption cultivated in both high-yielding high-input agricultural systems and in marginal low-input agricultural environments. It was domesticated 10,000 years ago in the Fertile Crescent (Badr 2000). Barley production in Australia is affected by increasing dryland salinity which severely limits growth and reduces yields (Rengasamy et al. 2003). Barley is notable in that it can maintain growth while accumulating high concentrations of Na^+ in its leaves (Munns et al. 1988). The high tissue tolerance of barley is likely to involve sequestration of Na^+ into intracellular vacuoles and the synthesis of compatible solutes that accumulate in the cytoplasm to balance the osmotic potential of the vacuolar Na^+.

13.4.3.1 Temperature Stress in Barley

Temperature stress has a strong effect on the growth and development of barley. Temperature can be divided into three ranges: (1) low temperature range, which includes cold and freezing; (2) optimum temperature range at which the plants grow and develop normally; and (3) high temperature range, which is associated with heat stress. The negative impact of climate change on crop yield has been established, with a projected increase in global temperature on the order of 4 °C by the late twenty-first century (IPCC 2014). High temperature (hereafter heat stress) affects the duration of the growing season, geographic distribution (Dawson et al. 2015), and malting quality of barley (Savin et al. 1996). The effects of heat stress depend on several factors, such as stress intensity (temperature in degrees) and duration and rate of temperature increase (Wahid et al. 2007). Symptoms of heat stress in barley include the reduction of yield and yield components, such as number of tillers per plant, spike length, and thousand grain weight, and a lower final starch concentration (possibly caused by irreversible inactivation of sucrose synthase) (Wallwork et al. 1998; Hogy et al. 2013; Abou-Elwafa and Amein 2016). Heat stress associated with high ambient temperatures is considered as one of the major abiotic stresses that limit crop growth and productivity (Shao et al. 2015). High temperature more than optimal adversely affects crop growth and reduces yield in multiple dimensions, viz., loss of moisture through early evaporation that results in poor crop establishment, and shrinks the optimum duration for vegetative growth, i.e., tillering and jointing that result in poor source development; chronic temperature during sink development results in premature flowering and accelerates grain filling period; loss

of pollen viability and embryo consequently leads to sterile florets with reduced grain number and size, promotes early senescence due to intensive chlorophyll loss, and ultimately reduces crop yield. However, yield losses vary and were associated with stage and duration at which plant encountered abnormal temperature, inherent genetic tolerance, mechanism involved in ameliorating the stress, and also availability of soil moisture due to its role in evaporative cooling of the canopy. Nevertheless, heat stress in barley is still an understudied topic, and further research is expected to take place due to climate change.

Barley, like other cereal crops, is particularly sensitive to heat stress during panicle development and meiosis, with high temperatures (over several days) causing abnormal pollen development and complete sterility (Sakata et al. 2000). Oshino et al. (2007) studied the effect of high-temperature injury on anther development in barley and observed a premature progression of early developmental stages and fate (e.g., progression to meiosis of pollen-mother cells). Gene expression and differentiation of anthers were studied under normal (20 °C day/15 °C night) and heat stress (30 °C day/25 °C night) conditions, revealing that genes that are active in the anthers under normal temperature conditions, such as H3, H4, and glycine-rich RNA-binding protein genes, were transcriptionally inhibited under heat stress (Abiko 2005). After anthesis, a field trial in Iran showed a 17% reduction in grain yield in barley under heat stress and significantly affected parameters, including the translocation of photosynthates to the grain, starch synthesis, and deposition in the developing grain (Modhej et al. 2015). Field trials have shown grain yield reduction caused by heat stress causing a higher penalty when temperature increased during stem elongation (Ugarte et al. 2007). In another field experiment using a panel of 138 spring barley genotypes, Ingvordsen (2015) corroborated the previously found decrease in grain yield (55.8%) caused by heat stress (Hogy et al. 2013). High temperature stress coupled with drought decreased the days to visible awns, days to heading, and days to ripe harvest, consequently negatively affecting crop growth and development of plants and resulting in a lower plant population m^{-2}, tillers $plant^{-1}$, plant height, and dry matter production m^{-2} (Hossain et al. 2012b). Stress disturbs normal homeostasis at cellular level and plant as whole in various ways (Wahid et al. 2007). Regarding photosynthetic machinery, it impairs RuBisCO activase in the Calvin cycle, photosystems I and II, drastic inhibition of carbon-dioxide fixation photophosphorylation capability, and inhibition of the electron flow from Q_A to Q_B (Berry and Bjorkman 1980; Cao and Govindjee 1990; Wahid et al. 2007).

Heat-resilient canopies are attributed by open canopy with glaucousness (light, bluish-gray waxy bloom on plant parts) and low carbon isotope discrimination (CID) that regulate stomatal conductance and maintain balance with photosynthetic capacity (Johnson et al. 1983; Condon et al. 2004). Rolled or erect upper leaves that help in deflecting and insulating direct solar radiation from the photosynthetic surfaces consequently result in cooler and longer viability of green canopy under warm dry conditions. Moreover, in this canopy system, a greater amount of water-soluble carbohydrates (WSC) are present in the stems compared to closed canopies (Van Herwaarden et al. 1998a) and that later remobilized to the grain during grain filling period and helps in modulating the adverse effect of heat stress in plants (van

Herwaarden et al. 1998b). However, the adverse effect of heat stress can be minimized by avoiding it either through adjusting sowing time or by early maturing genotypes at the most sensitive stages such as reproductive or grain-filling periods (Prasad et al. 2008; Hossain et al. 2012a). Even breeding for early cultivars to incorporate escape mechanism against heat stress during grain filling is not a viable option as it results in shorter phenophases and lower accumulation of biomass, hence poor sink size. There is a need to optimize flowering duration and time, which more or less depends upon sowing time and prevailing environment condition but keeping flexibility in sowing time (Hunt et al. 2018)

13.4.3.2 Freezing Stress

Low-temperature stress includes cold (also known as chilling, above freezing point temperature) and freezing (below freezing point temperature) and has a severe effect on small-grain crop plants in temperate climates (Kosova et al. 2011). In regions such as southern Australia, where winter temperatures are not low enough to cause freezing damage at the vegetative stage, radiation frost constitutes a problem at the reproductive stage. Radiation frost occurs during clear nights when the plant canopy is receiving less heat than what is radiated away, causing temperatures to drop below zero. Yield is severely affected by radiation frost as a result of floret and spike abortion as well as damage to developing grains (Reinheimer et al. 2004; Zheng et al. 2015). Freezing tolerance in barley is a polygenic trait with complex interactions between loci, genotype, and environment (Busconi et al. 2001; Ceccarelli et al. 2010). This tolerance associated with freezing invariably affects metabolic processes such as redox reactions, photosynthesis, carbohydrate metabolism, transcription, and protein expression. Various types of proteins are involved in development of defense mechanism such as late embryogenesis abundant and heat shock proteins, photosynthetic electron transport chain components, chaperones, and reactive oxygen species—destroyer enzymes (Gharechahi et al. 2014; Janmohammadi et al. 2015). Proteomic study showed that higher activity of RuBisCO activase and greater abundance of RuBisCO (large and small subunits) and oxygen-evolving enhancer proteins, viz., ferredoxin-NADP reductase and cytochrome P450-dependent fatty acid hydroxylase, were associated with greater freezing tolerance level in barley. Moreover, lower relative level of hypothetical ATP synthase beta subunit, mitochondrial protein AtMg00810, and small subunit methyltransferase G of ribosomal RNA are more important for imparting freezing tolerance, and H_2O_2 acts as a signalling molecule involved in the commencement of cold-induced acclimation in barley (Golebiowska-Pikania et al. 2017). These types of study further amplified with strategic screening with double haploid (DH) lines that not only produce totally homozygous lines but also uncover the inherent genetic diversity as a whole (Golebiowska-Pikania et al. 2017).

Salt Stress: Accelerated development of saline soil is a severe threat for sustainable agriculture. A serious concern is that in the future, salinization of soil will increase, and now it is reducing the agricultural area by 1–2% every year in the arid

and semiarid regions, and it is projected that by the year 2050, more than 50% of cultivable soil will be affected (Vinocur and Altman 2005). Barley is regarded as salt tolerant among crop plants; however, under severe stress scenario, its growth and development are severely affected by ionic and osmotic stresses (Mahmood 2011). Soil salinity is a major constraint on agriculture and impacts food security and political stability. Currently, 1128 Mha, including 20% of irrigated lands (http://www.fao.org/water/en/), is estimated to be affected by soil salinity. The largest area, of 189 Mha, is located in the Middle East (Wicke et al. 2011). These numbers are expected to increase due to climate change and poor irrigation practices (Hayes et al. 2015). Qadir (2014) estimated that an annual economic loss of US$27.3 billion is due to soil salinity. Barley varieties grown in these marginal areas have to be tolerant to soil salinity. Salt tolerance in cereals is known to be associated with the control of shoot Na+ content; tolerant lines have more efficient systems to exclude sodium from their cells (Dubcovsky et al. 1996). Widodo et al. (2009) reported that metabolite changes in response to the salt treatment also differed in the two cultivars. After 3 weeks of salt treatment, Clipper ceased growing, whereas Sahara resumed growth similar to the control plants. Sahara, the better-adapted cultivar, displayed tolerance to high internal salt concentrations without apparent cell damage suggesting that this cultivar may have mechanisms either to maintain a higher K^+/Na^+ ratio in the cytoplasm through compartmentation of Na^+ into the vacuole or by increasing the metabolite levels to cope with the increased osmotic potential. Salt stress in plants induced through development of soil salinity results in water stress, nutritional and hormonal imbalance, specific ion toxicity, and disturbance in homeostasis of Na^+ and Cl^- ions and induces reactive/active oxygen species (ROS/AOS) production in chloroplasts that oxidizes biological molecules, such as DNA, proteins, and lipids (Fadzilla et al. 1997; Meneguzzo et al. 1999; Steduto et al. 2000; Liang et al. 2003; Gunes et al. 2007; Daneshmand et al. 2010). That inhibits rate of photosynthesis in plant, protein building, and lipid metabolism (Paul and Lade 2014) that subsequently leads to death of plant cell (Omar et al. 2009). Wakeel et al. (2011) suggested that the Na^+ toxicity affects plant growth and increases Na^+/K^+ ratio, and thus, displacement of K^+ by Na^+ in the plant cell affects the activity of plasma membrane (PM) H^+-ATPase. Addition of K^+ to NaCl and water-deficit stressed plants reduced the Na^+ and increased K^+ content within leaves. Na^+/K^+ ratio increased with increasing salt doses. Plants respond in many ways to salinity and at a number of levels (Munns and Tester 2008). It is expected that exposure of plants to salinity will also result in a wide range of metabolic responses. While changes in levels of several metabolites following salt stress are well documented, less well documented are the changes that occur in the metabolome overall (Zuther et al. 2007; Sanchez et al. 2008a, b).

Under salt stress condition, there is decrease in plasma membrane H^+-ATPase activity, plasma membrane fluidity in the leaves, and glutathione content, while the ratio of phospholipids to proteins in plasma membrane vesicles increased (Liang et al. 2006). Both superoxide dismutase (SOD) and catalase (CAT) activities decline in plants (Singha and Choudhuri 1990), while malondialdehyde (MDA) accumulates rapidly (Fadzilla et al. 1997) that results in plasma membrane permeability.

Both enzymatic (superoxide dismutase, catalase, ascorbate peroxidase, peroxidase) and non-enzymatic (glutathione, ascorbic acid, and carotenoids) systems are known for scavenging AOS in plants (Breusegem et al. 2001). In leaves, under salt-induced water-deficit conditions, shoot growth is reduced, and there is considerable alteration in soluble carbohydrate, proline, total phenolic compounds, antioxidant activity, Na^+ content and K^+ homeostasis or Na^+/K^+ ratio, leaf photosynthetic pigments and leaf soluble protein content as provoked by oxidative stress. General metabolic adaptation, which enables plants to cope with water or osmotic stress, involves an increased synthesis of osmoprotectants, such as proline and soluble sugar that not only contribute to osmoregulation but also protect the structure of different biomolecules and membranes (Yancey et al. 1982; Hare et al. 1998) or act as free-radical scavengers that protect DNA from the damaging effects of ROS (Ashraf and Foolad 2007). Proline accumulation has been reported to counterbalance the deleterious effects of water deficit and salinity and act as an organic nitrogen reserve that recovers the system quickly (Sairam and Tyagi 2004).

13.4.3.3 Heavy Metal Toxicity

Al resistance usually follows the order: rice = rye > wheat > barley, although genetic variation within species also exists (Kochian 1995). Toxic aluminum (Al) cations solubilized by the acidity cause rapid inhibition of root elongation and root growth subsequently and reduce uptake of water and nutrients. Prominent pathways for detoxification include chelation or exclusion of Al at the apoplast via rhizosphere alkalinization (Degenhardt et al. 1998) and release of organic acids (Ma et al. 1997) and phenolic compounds (Kidd et al. 2001). Citrate was secreted from the root apices, which helped in excluding Al and thereby detoxifying Al. A positive correlation was found in response to Al stress between citrate secretion and Al resistance and a negative correlation between citrate secretion and Al content of root apices (Zhao et al. 2003). ALMT1 gene encoded a malate transporter that is associated with malate efflux and Al tolerance, which is known for its ability to confer effective tolerance to acid soils through a transgenic approach in crop species (Delhaize et al. 2004). Al stress significantly increased the Ca^{+2} efflux and inhibited the K^+ efflux and ATPase activities, especially in the Al-sensitive genotypes. While higher Al and low-pH tolerant genotype XZ16 (Tibetan wild barley) showed higher ability of H^+ uptake, rhizospheric alkalization, lower Al-induced Ca^{2+} efflux, and ATPase inhibition (Liu et al. 2018). Both copper (Cu) and manganese (Mn) in excess inhibit respiration, negatively affect nitrogen and protein metabolism, cause reduction of chlorophyll contents, and inhibit photosynthetic functions in leaves (Foy et al. 1978). Cu in excess blocks the photosynthetic electron transport, inhibits photophosphorylation, and decreases membrane integrity (Maksymiec et al. 1994). Alteration of the source-sink relationship will consequently diminish requirement for products of photosynthesis and downregulation of the Calvin cycle enzymes (Maksymiec 1997). Mn toxicity is observed in strongly acidic soils, reducing conditions created by organic matter accumulation, compaction, or flooding; in some

soils after unequilibrated fertilization (e.g., with high rates of superphosphate or nitrate); and in soils deficient in Ca, Fe, Mg, or P. Cu or Mn toxicity was most probably the consequence of depletion in low-molecular antioxidants as a result of their involvement in detoxification processes and disbalance in antioxidative enzymes. Cu excess affected mainly the non-protein SH groups, while Mn influenced the ascorbate content. Ascorbate peroxidase activity was diminished under Mn toxicity and was not changed under Cu excess, protein carbonylation, a lower superoxide dismutase activity, and elevated catalase and guaiacol peroxidase activities (Demirevska-Kepova et al. 2004). Cadmium (Cd) interferes with many cellular functions mainly by complex formation with side groups of organic compounds such as proteins resulting in inhibition of essential activities that results in poor antioxidant defense systems, which cause oxidative damage, lipid peroxidation, and membrane leakage in plants.

13.4.3.4 Drought Stress

It is defined as water availability below that required for maximum crop yield and is the main factor limiting crop production worldwide. It represents a permanent constraint to agricultural production in many developing countries, although drought also causes great yield losses in developed agricultures. Drought events are often associated to high temperatures which impose an additional level of stress to plants. Samarah and Alqudah (2011) reported that late-terminal drought stress during grain filling has recently become more common in the semiarid Mediterranean region, where barley (*Hordeum vulgare* L.) is grown as an important winter cereal crop. Little information is available in the literature about the effect of late-terminal drought stress on seed germination and vigor of barley. Drought stress reduced grain yield of barley. Grain yield was correlated positively with leaf gross photosynthetic rate and negatively with leaf osmotic potential. Late-drought stress had no effect on standard germination but reduced the germination after the accelerated aging test. These data suggested that late-terminal drought stress had a greater effect on seed vigor than standard germination in barley. Drought is becoming more common worldwide, causing devastating effects on crop production (Ludlow and Muchow 1990). Several reports have suggested that drought stress during seed development of barley reduced leaf photosynthetic rate (Samarah 2004; Masoud et al. 2005), decreased grain filling duration (Sanchez et al. 2002; Samarah 2004), and enhanced plant maturity, resulting in a serious reduction in grain yield and yield components (Forster 2004; Samarah 2004). In addition to its effects on the physiological processes in plants, drought stress during seed development can reduce seed germination and vigor. In barley, the unsuitable weather conditions in 3 out of 7 years studies resulted in production of low seed vigor (ranging from 61% to 86%) as compared with the high seed vigor (exceeded 94%) of the seeds produced in the remaining 4 years (Chloupek et al. 2003). Samarah and Alqudah (2011) reported that drought not only reduced the germination in the small seed size category, which consisted of shriveled, misshapen, and hard seeds, but also reduced the vigor.

13.4.3.5 Waterlogging

Optimum soil gas exchange is severely hampered under waterlogging condition that results in alteration of soil redox potential subsequently and availability of plant nutrients. Prolong submerged conditions result in depletion of soil free oxygen, while accumulation of greater amount of CO_2 was associated with higher root and microbial respiration (Bailey-Serres and Voesenek 2008). Furthermore, this hypoxia stress with reduced redox potential limits ATP synthesis production in plants (Barrett-Lennard 2003). In addition to this, the concentration of some minerals due to more solubility on reduction (Mn^{4+} to Mn^{2+}, Fe^{3+} to Fe^{2+}, SO_4^{2-} to H_2S) reaches toxic levels accompanied with other organic compounds formed by anaerobic respiration of plant roots and microbes. Physiologically, plants respond at various levels and in variable intensity with reduced stomatal conductance, diminishing leaf water potential, altered membrane transport, accelerated root senescence, stubby root and shoot, and, ultimately, death of plant under severe waterlogging conditions (Barrett-Lennard 2003). Moreover, waterlogging does not come solely as the phenomenon largely in the proximity of salinity where intensive irrigation, sea water intrusion especially in coastal environments, and rise of saline water multiply its effect on plants (Barrett-Lennard 2003) and thus reduce crop yields about 80%. Under combined scenarios (salt and waterlogging), there were about twofold escalation of Na^+ and 40% reduction of K^+ in leaves of barley, and also the effect was more pronounced in sandy loam as compared to vermiculite. Just for 2 weeks, the above scenarios significantly reduced plant biomass, maximal quantum efficiency (PSII), and relative water and chlorophyll content of barley varieties (Zeng et al. 2013). Another study advocates positive correlation between Mn^{2+} toxicity to impart waterlogging stress tolerance in barley. The study revealed that under high Mn^{2+} level, most waterlogging genotypes showed chlorophyll content above 60% while sensitive genotypes possessed less than 35% and suggested that traits conferring tolerance to Mn^{2+} toxicity also impart tolerance to waterlogging and thus accelerate breeding for waterlogging tolerance in barley (Huang et al. 2015). Cultivated barley shows a genetic diversity in waterlogging tolerance, and this tolerance varies according to life stage, with barley being more susceptible to waterlogging at pre-emergence, seedling growth, and reproductive stages (Setter and Waters 2003). Although barley is sensitive to Mn toxicity, a large genetic variation in Mn tolerance exists among cultivated genotypes (Hebbern et al. 2005).

13.4.3.6 Lodging

The process by which shoots of winter or summer cereals are displaced from their vertical orientation is called lodging. In cereals such as wheat and barley, lodging is most likely to occur during the 2 or 3 months preceding harvest, usually after ear or panicle emergence, with the result that shoots permanently lean or lie horizontally on the ground. Lodging can be caused by the buckling of stems (stem lodging) or displacement of roots within the soil (root lodging) (Tams et al. 2004). In stem lodg-

ing, roots are held firm in a strong soil where the wind force buckles one of the lower internodes of the shoot. Root lodging becomes more likely when the anchorage strength is reduced by weak soil or poorly developed anchorage roots. The effect is a reduction in crop yield by up to 80%, with further losses in grain quality, greater drying costs, and an increase in the time taken for harvesting

Light Stress: Plants grown under high light intensity showed reduction in quantum yield of photosystem II, photosynthetic electron transport capacity, and photochemical quenching. Photosystem I was found more stable against photo-inhibition associated with the cyclic electron flow (Quiles and Lopez 2004;Kalaji et al. 2012). Both high and low light stresses reduced activity of photosystem II in barley seedlings, while response varies with salt type, its duration, and genotype. Moreover, chlorophyll-a fluorescence performance index parameter is quite sensitive under low and high stress and can be recommended for early detection of light stresses. A study was conducted to understand source-sink dynamics in spring barley with an objective to quantify grain abortion, post-anthesis assimilation in response to reduction in post-anthesis incident radiation, and grain weight heterogeneity in relation to post-anthesis assimilation per unit grain number. Two treatments were superimposed at post-anthesis in barley as shaded (59% of incident radiation on crop) and unshaded, under former one barley mean grain weight reduced by 12–16%, grain yield by 19–20%, and harvest index by 5–6% with shading from 14 days after anthesis until harvest maturity. Moreover, shading reduced grain filling rate about 23–27% but more filling in central spikelets than at distal or basal locations on the ear, while the situation failed to produce any significant effect on grain number and duration of grain filling (Kennedy et al. 2018).

13.4.3.7 Nutrient Stress

Crop nutrition is a key determinant of yield potential. An adequate supply of essential macro- and micronutrients is required for crops to achieve sufficient vegetative growth to ensure the development of nutritious grain. Fertilizer is a major input cost for farmers but is nevertheless often applied in excess of a crop's requirements. They may be leached from the soil or lost to volatilization (Sylvester-Bradley and Kindred 2009). The impact of this "lost N" is of critical concern as a source for major economic loss as an environmental pollutant that leads to release of potent greenhouse gases and eutrophication of aquatic environments (Zhang et al. 2015). Insufficient availability of N and phosphorus (P) has the greatest impact on yield potential of crop plants (Hawkesford et al. 2012). One of the important aspects for P uptake is the plants interaction with mycorrhizae. Selecting varieties of barley and mycorrhizae that maximize P uptake in an agricultural environment is a possible strategy to improve Nutrient Use Effeciency (NUE) (Grace et al. 2009). Phosphorus (P) is an energy source of plants, and under deficient condition, it results in poor root and shoot growth. Most of applied P fixed reversibly or irreversibly on reaction with aluminum and iron oxides especially under acidic soils, transformed by microbes as energy source for their growth (Jez et al. 2016), and remaining through

drainage and runoff leads to serious environmental concerns such as water eutrophication (Andersson et al. 2013). However, plants show various mechanisms such as secretion of organic acids, viz., malate and citrate, into rhizosphere to solubilize and enhance their uptake via ligand exchange (Ding et al. 2016). Even then phosphorus use efficiency in plants is generally about 30%. Hence, there is a need to intensify breeding strategy and develop varieties or screening traits with high phosphorus acquisition and use efficiency.

13.4.4 Resistance to Biotic Stresses

The development of advanced modern varieties of barley led to the replacement of locally adapted landraces, thereby narrowing the genetic base of the crop. The analysis of both pedigrees and molecular data generated by different researchers showed that many of the cultivars grown share related pedigrees and there is high similarity between some varieties according to their origin and pedigree with a preference to one-parental inheritance in certain cases (martin et al. 1991;Sjakste et al. 2003). Similar agronomic and end-use characteristics preference by the consumers reason this aspect, favoring mostly the crosses to be performed among elite genotypes of limited number. Such breeding programs ultimately lead to narrowing of the genetic diversity in barley.

In terms of breeding, genetic diversity is paramount due to its ability to make progress toward a breeding target and an important source of de novo beneficial alleles. In barley, the primary germplasm pool is constituted by cultivated barley (including landraces, breeding lines, and cultivars) and *H. vulgare* ssp. *spontaneum*; the secondary germplasm pool is of a single species *Hordeum bulbosum* L. (bulbous barley grass), and the tertiary germplasm pool is comprised of about 30 different *Hordeum species* (Nevo 1992). The largest component in barley germplasm is constituted by landraces (44% of the 290,820 barley accessions held in gene banks around the world).

The pre-breeding approach is the most promising for linking genetic resources with breeding programs. The process involves trait identification, gene discovery, developing markers, and screening population using conventional and molecular tools for generating valid information. The information displayed by researchers on different characteristic features of a breeding line is expected to have merits for its further inclusion in vivid breeding programs designed by the breeders. Pre-breeding programs not only generate new base populations for breeding programs but also assist in identifying heterotic combinations for hybrid programs. Internationally, International Center for Agricultural Research in the Dry Areas is one of the premier institutes working in exploiting locally adapted germplasm, mixtures, and landraces; barley improvement; and conservation and also supplies germplasm for different breeding programs.

The pre-breeding work on barley landraces and wild progenitors has been done by many researchers. *Hordeum vulgare* ssp. *spontaneum* C. Koch has been

reported to be a valuable source of new genes for breeding including grain protein (Jaradat 1991), drought tolerance (Baum et al. 2003), resistance to powdery mildew (Dreiseitl and Dinoor 2004), earliness (Ogrodowicz et al. 2017), and cold tolerance (Grossi et al. 1998). Jin et al. (1994) identified several potential sources of resistance to leaf rust in *H. spontaneum* accessions (PI 354937, PI 355447, PI 391024, PI 391069, PI 391089, PI 466245, and PI 646324). Papa et al. (1998) studied different landraces of barley from Italy and confirmed high variability within landrace populations, underlining the importance of landraces as a source of genetic variation. Sanjaya Gyawali et al. (2017) identified eleven potential donors of stripe rust seedling resistance from 336 barley genotypes against six races. Several barley leaf rust all-stage resistance genes conferring high level of resistance, including *Rph1,Rph19*(Golegaonkar et al. 2009), *Rph21* (Sandhu et al. 2012), and *Rph22* (Johnson et al. 2013), have been characterized. For powdery mildew, Jahoor and Fischbeck (1987) and other researchers have reported different sources for its resistance. The potentially different source of resistance against the important biotic stresses in barley has been divulged by different researchers (Table 13.2).

13.5 Breeding Techniques

Barley is a highly self-pollinated cereal crop, so most of the breeding methods used in self-pollinated crops are used in barley breeding programs. The usual procedure of selecting the parents and their hybridization and advancing the segregating generations is followed. The methods for advancing the generations may differ from one trait to another. Also backcrossing and production of homozygous lines using doubled haploidy techniques are common in barley. Mutation breeding and development of hybrids for barley improvement are also reported. Recently, molecular marker-assisted breeding has also been reported in barley for the traits of economic importance, especially malting quality. Here, the methods are described in some detail.

13.5.1 Bulk Method

Bulk handling of crosses has been used for many years as an inexpensive way to grow early generation materials with or without selection. At the end point of bulk breeding systems, selections are made and tested as individual lines. Bulk breeding systems are well adapted to mass selection. For example, screening for winter hardiness and kernel plumpness can be done by mass selecting bulk materials.

Table 13.2 Variability studies with respect to different disease resistance in barley

Reference	Variability studies by different researchers for disease resistance
Stripe rust or yellow rust	
Sethi et al. (1973)	Tested 288 lines and varieties for reaction to *Puccinia striiformis* and reported the six-rowed line BHS4-7-1 as immune and 51 as fairly resistant
Mathur and Siradhana (1990)	Screened 700 cultivars and lines to a natural epiphytotic of the *Puccinia striiformis*, 15 remained free from infection and 11 showed only traces of the rust and were regarded as resistant
Chen et al. (1995)	Reported barley genotypes—Hor 1428, Hor 2926, Hor 3209, BBA 2890, Abyssinian 14, Grannelose Zweizeilige, Stauffers Obersulzer resistant to all races of *Puccinia striiformis hordei f. sp. (Psh)* that have been detected in the United States and Heils Franken, Emir, Astrix, Hiproly, Varunda, Trumpf, Mazurka, Bigo, Cambrinus, BBA 809, and I 5 were resistant to some races, i.e., they showed differential reactions to the races
Yadav and Kumar (1999)	Evaluated 168 accessions of which seven lines were reported to be resistant
Castro et al. (2003), Toojinda et al. (2000)	Identified a number of provisionally designated genes conferring resistance to stripe rust from barley germplasms
Verma et al. (2016)	Identified 12 stripe rust-resistant genotypes against five *PSH* races in India from the high-input barley breeding program of the International Center for Agricultural Research in the Dry Areas (ICARDA)
Gyawali et al. (2017)	Tested 336 barley genotypes for barley stripe rust resistance. Genotypes—AM-14, AM-177, AM-37, AM-120, AM-300, AM-36, AM-103, AM-189, AM-291, AM-275, and AM-274 showed resistance response to all the races tested at seedling and adult-plant stages
Gyawali et al. (2018)	Reported 89 barley genotypes with higher level of APR which showed susceptible IT to at least one *PSH* race at seedling stage
Verma et al. (2018)	Screened 336 barley genotypes consisting of released cultivars, advanced lines, differentials, and local landraces from the ICARDA barley breeding program for seedling and adult-plant resistances to barley stripe rust pathogen, of which twelve genotypes (ARAMIR/COSSACK, Astrix, C8806, C9430, CLE 202, Gold, Gull, Isaria, Lechtaler, Piroline, Stirling, and Trumpf) were reported to be resistant at the seedling and adult-plant stages. Forty-five genotypes showed adult-stage plant resistance (APR) in the field
Leaf rust or brown rust	
Dill-Macky et al. (1991)	Screened 370 disease-free barley selections with *Puccinia graminis* var. *tritici* in the field and selected lines further evaluated with *Puccinia graminis* var. *secalis*. Rust development was minimum in lines Q21861, Q21928, and Q21972
Jin et al. (1994)	Evaluated cultivated and wild barley for resistance to pathotypes of *Puccinia hordei* with wide virulence. Five *Hordeum vulgare* and 167 *Hordeum spontaneum* accessions were identified as possible sources of new genes for leaf rust resistance

(continued)

Table 13.2 (continued)

Reference	Variability studies by different researchers for disease resistance
Amgai et al. (2016)	Screened 241 Nepalese barley gene pool accessions for leaf rust resistance and reported 109 to be promising for disease resistance
Steffenson et al. (2016), Steffenson et al. (2017)	Identified barley landraces from Switzerland carry a high frequency of stem rust resistance and reported the genes underlying this resistance as Rpg1 and the rpg4/Rpg5 gene complex
Elmansour et al. (2017)	Identified 13 accessions to carry *Rph23* and ten accessions with moderate APR lacked *Rph23*, indicating that they likely carry new uncharacterized APR genes
Stem rust or black rust	
Jedel et al. (1989)	Studied seedling and adult plant resistance in controlled environments with five races (C5, CIO, Cl7, C25, and C35) in 244 accessions from Ethiopia. The accession PI382313 was resistant or moderately resistant to all six races. The gene was identified as Rpg3
1. Dill-Macky et al. (1990); Dill-Macky et al. (1992)	Reported the accession Q21861 (PI 584766) to carry high levels of resistance at the seedling and adult-plant stage to race QCCJB
Dill Macky et al. (1992)	Identified accession Q21861 a CIMMYT line with unknown pedigree as a resistant source to stem and leaf rusts
2. Steffenson (1992)	Reported the first stem rust resistance gene Rpg1 (originally designated T) from the Swiss landraces of Chevron (PI 38061) and Peatland (CIho 2613)
Fox and Harder (1995)	Evaluated 22 barley (*Hordeum vulgare* L.) cultivars and lines for resistance to stem rust race QCC in field trials over a 3-year period. Rust severities ranged from 17% to 58% between 14 different cultivars and lines which carried the stem rust resistance gene *Rpg1*, indicating that factors other than *Rpg1* conferred resistance
Fetch and Dunsmore (2004)	Founded two accessions (119Y4 and 212Y1) resistant to pathotype Pgt-QCCJ of stem rust
Steffenson and Jin (2006)	Identified several sources of resistance to race TTKSK in landraces (*H. vulgare*) and wild barley (*H. vulgare* subsp. *spontaneum*) accessions
Fetch et al. (2009)	Reported a novel source of resistance to race QCCJ from 212Y1
Derevnina et al. (2014)	Evaluated 82 Australian and five exotic barley cultivars at the seedling stage for resistance to the Australian stem rust pathotype 98-1,2,3,5,6. Although most of these cultivars exhibited mesothetic (mixed infection type) reactions that were associated with a high level of chlorosis, two ('O'Connor' and 'Pacific Ranger') were highly resistant
Steffenson et al. (2016)	Reported 73 Switzerland barley landraces with remarkably high frequency (>43%) of resistance to the virulent races of TTKSK and QCCJB and reported this resistance to be due to the rpg4/Rpg5 gene complex
3. Steffenson et al. (2017)	Evaluated wild progenitor (*H. vulgare* subsp. *spontaneum*), to pathotype TTKSK. Only 13 (1.4%) wild barley accessions exhibited consistently HR to MR reactions across all experiments

(continued)

Table 13.2 (continued)

Reference	Variability studies by different researchers for disease resistance
Powdery mildew	
Negassa (1985)	Reported Mlo-resistant barley landraces collected from South Ethiopia
Jahoor and Fischbeck (1987), Repkova et al. (2006)	Identified number of accessions of *H. vulgare* ssp. *spontaneum* for useful resistance to powdery mildew
Czembor and Czembor (2001)	Screened 48 populations of barley landraces collected from Morocco for resistance to powdery mildew and identified 20 populations as resistant source
Urrea et al. (2005)	Identified an accession CIho 7595 to be resistant to powdery mildew, net blotch, and spot blotch
Pandey et al. (2000)	Evaluated hull-less barley landraces from the Himalayas of Nepal for resistance to barley powdery mildew and other diseases. The result highlighted a differential resistance pattern indicating the presence of We, U2, /St, U2/Ly, We, Kw, La/Ha/Mlp/Mla22/Mla27 types of powdery mildew resistance

13.5.2 Composite Crosses

Composite crosses result from combining a number of single crosses into one large mixture or composite. It utilizes large germplasm pools with minimum time and cost. Composite crosses are an efficient way to do long-term breeding, especially when genetic male sterility is used to facilitate the mating system. When a composite cross is grown in the intended-use area long enough to allow natural selection to influence gene frequencies, most of the selected plants are well adapted to that area. Generally, objectives in composite cross breeding should be long term in nature to encourage potential recombination of many factors, and thus, germplasm should be as broad based as possible.

13.5.3 Male Sterile-Facilitated Recurrent Selection

In the past, natural or mass selection has been the predominant method for improvement of composites. Recently, selected mating within composites has been utilized to achieve certain objectives. The term male sterile-facilitated recurrent selection (MSFRS) was first used by R.F. Eslick to describe this breeding technique in barley. This procedure has been used to develop short-stemmed, large-seeded barley populations, disease-resistant populations, and winter-hardy populations. This breeding method theoretically provides a continuing opportunity for interaction among alleles at heterozygous loci.

13.5.4 Pedigree Breeding Method

Pedigree breeding with various modifications has been widely used in barley breeding programs. The yield test can be carried out as early as in F_5 or delayed until the F_7 or later, depending on breeding performance and the genetic variability present in the cross. Optimum F_2 and F_3 population sizes will vary and should be adjusted based on the objective and the nature of the particular cross. The pedigree method works well for characters that can be easily identified in the early segregating generations. However, for characters with low heritability, the pedigree system can be a form of pseudo-random selection, a situation which must be carefully guarded against. The pedigree breeding system can easily be modified in any stage of selection. Two such modifications were given by Lupton and Whitehouse (1957). In F_2 progeny method, similar numbers of F_3 families are selected from each of several crosses and entered in yield trials in the F_4 and F_5 generations. Final plant selections are made in the F_6 generation. This method allows selection for yield up to 2 years before final reselections are made. The second modification is pedigree trial method in which F_3 plants within F_3 families are selected and grown as separate F_4 lines. A selected F_4 line in each F_3 progeny group is advanced for further pedigree work, and the remaining lines are bulked to provide seed for F_5 yield trials. Similar trials are grown in the F_6 and F_7 generation with seed provided each year by superior progeny rows and from the preceding trial. With more advanced stages of testing, the progeny rows and the yield test material become very similar, so that no additional plant selection is necessary. Obviously, the time span of testing is reduced in the pedigree trial method compared to the F2 progeny method.

13.5.5 Backcross Breeding

The backcross breeding method as first suggested by Harlan and Pope (1922) involves repeated backcrossing to one parent after an initial cross, with the intent of recovering the recurrent parent with the feature added from the donor parent. This breeding system is well suited to simply inherited traits controlled by one or two major genes. Backcrossing is also well suited in the development of isogenic lines for genetic studies.

13.5.6 Single Seed Descent

The single seed descent (SSD) breeding method was proposed by Goulden (1939) as a procedure for obtaining a high level of homozygosity while maintaining maximum genetic variation in self-pollinating species. The selection of single seeds from each plant as the generations are advanced from F_2 to the desired level of homozygosity is the basis for the term single seed descent.

13.5.7 Haploid Breeding Method

The production of a high frequency of viable haploids that can subsequently be doubled with colchicine is an important development in barley breeding because it allows the recovery of homozygous inbred lines in single generation. Several methods are available for haploid production in barley. The first to be reported was the chromosome elimination method of Kasha and Kao (1970). Haploids of *H. vulgare* are produced as a result of gradual elimination of *H. bulbosum* chromosomes from hybrid embryos of the two species. This technique, known as the bulbosum method, is the most efficient and used one in barley for the development of homozygous lines.

13.6 Biotechnology-Based and Marker-Assisted Approaches

Modern variety breeding aims to develop cultivars with high adaptability and elasticity so that they may be successfully grown in widely different environments. Therefore, cultivars developed under continental climatic conditions as prevalent in large parts of Germany should also be competitive in other European countries and elsewhere. Such successful modern cultivars represent optimal genetic complexes determining the necessary trait combinations providing high crop stability and performance. Actual German spring barley cultivars are characterized by such optimal trait compositions, for example, ‘Streif’ or ‘Tocada’, combining broad resistances against abiotic and biotic stresses with high yield potential and superior malting quality.

With the advent of molecular markers, the ability to utilize wild relatives in crop improvement has greatly improved relative to conventional approaches (Xiao et al. 1996). In many breeding programs, molecular breeding complements conventional breeding, as these new tools allow breeders to identify and follow desired alleles through marker-assisted selection (MAS) in the breeding/selection process (Rao et al. 2007). The progress in biotechnology has opened up enormous possibilities, both for introgression of specific traits and for base broadening in pre-breeding. Even the large size of the barley genome has not restricted the progress in molecular mapping. The techniques rendering this development initially included expressed sequence tag (EST) resources for gene-based markers, development of a commercial micro-array with 23,000 barley genes, and the synthesis of DArT markers for specific chromosomal regions. Comprehensive consensus maps provided means to select markers for chromosomal regions and allow comparative mapping by exploring information available for other grasses. Then high-throughput genotyping (Single Nucleotide Polymorphism, DArT) increased data generation speed. Increasingly genomic analysis of germplasm can be outsourced to commercial parties, and in-house emphasis is on interpreting the returned data through bio-informatics. Consortia efforts are in progress for the sequencing of barley genome (http://barleygenome.org), which will facilitate rapid cloning of genes and increase the

number of markers available for mapping quantitative trait locus (QTL) and implementing marker-assisted selection. The barley genome is one of the largest in cereal crops, and a substantial body map of genetic and genomic resources has been produced (Martin et al. 2017) as high-quality reference genome assembly for barley.

Transformation efficiencies in barley continue to increase, and this is allowing the demand for an evaluation of gene function using transgenic tools. However, the pace of gene discovery is also increasing, with the availability of more sequenced crop genomes and improved genomics tools, meaning that even more genes will need to go through a transformation pipeline to allow the study of gene function (Harwood 2012). A range of tools are available to help achieve the level and specific pattern of transgene expression required, but there are still gaps in the range of promoters available for use in barley that need to be addressed. However, further advances in this technology are required before it can be used routinely in barley. A key remaining challenge is the genotype dependence of most wheat and barley transformation systems, and this continues to restrict the application of the technology. It is, however, likely that, by understanding and manipulating plant genes important in either the plant regeneration process or in susceptibility to Agrobacterium, it will be possible to address issues of genotype dependence and to improve transformation efficiencies further. Marker-based technologies together with doubled haploid technologies and "speed breeding" have almost halved the time of variety development in some barley breeding programs (Hickey et al. 2017). Genome-wide association studies (GWAS) with information on linkage disequilibrium (LD) have revolutionized the QTL studies to unravel the genetic architecture of complex agronomic traits. New QTL for biotic stresses like net blotch (Amezrou et al. 2018; Gyawali et al. 2018b), spot blotch (Gyawali et al. 2018a), and stripe rust (Visioni et al. 2018) and grain micronutrients (Gyawali et al. 2017) have been discovered in global collections at ICARDA in association with other institutions. As an outcome, genomic selection using entire genome information is proving the latest tool in making breeding more precise and faster. Therefore, the strategy for future barley breeding should be built on in-depth knowledge of the barley genome and promote the use of both older and modern proven technologies to achieve the final goals more rapidly.

Besides numerous health claims attributed to barley in general (Ames and Rhymer 2008), several studies have demonstrated the specific health-promoting properties of β-glucans (Wood and Beer 1998; Newman and Newman 1992). β-glucans have been implicated in lowering plasma cholesterol, improving lipid metabolism, and reducing glycemic index and the risk of colon cancer (Behall et al. 2006; Brennan and Cleary 2005; Keenan et al. 2007). Pearling by-products have interesting amounts of bioactive compounds (dietary fiber and β-glucans) and could therefore be proposed as potential ingredients for the manufacture of functional food (Marconi et al. 2000). Development of new varieties (Fig. 13.2) with desired traits offers farmers greater flexibility in adapting to climate change, including traits that confer tolerance to drought, heat, and salinity, and early maturation in order to shorten the growing season and reduce the crop's exposure to risk of extreme weather events (Lybbert and Sumner 2010). Barley farmers pursue a wide range of

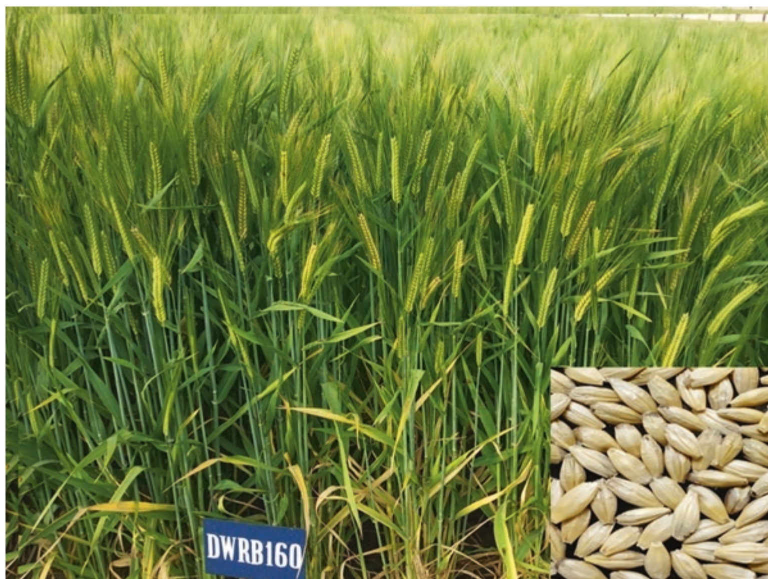

Fig. 13.2 DWRB 160—a recently released malt barley variety in India

crop and livestock enterprises that vary not only within but across the major agro-ecological zones. Barley certainly contributes to their livelihoods as well as for their livestock in terms of grain, straw, and grazing in the dry environments. The additional income from the cultivation of malt barley with private partnership is an important factor in promoting barley cultivation in East Africa and South Asia. Understanding farmers' response to climatic variation is, therefore, crucial in designing appropriate copping strategies to climate change for poor countries, which are highly vulnerable to the effects of climate change.

13.6.1 Evolution of Breeding Methods

13.6.1.1 Acceleration of Barley Breeding via Haploidy

Combinations of different resistance genes or the introgression of novel resistances from nonadapted germplasm into adapted cultivars' background are classically achieved by sexual recombination, that is, crosses between selected parental lines followed by phenotypic selection in the segregating offspring. In this case, the success of breeding entirely depends on extensive field and/or greenhouse tests for resistance to the respective pathogen(s). However, since barley is damaged by many pathogens, which often show a rapid adaptation to their hosts' resistance, breeding for resistance is a very complex task, and the identification of desired recombinants by phenotypic selection, for example, in pedigree selection schemes, has almost

reached the limits of manageability. Thus, methods of plant biotechnology like anther and microspore culture allowing the rapid production of homozygous doubled haploid (DH) lines and cultivars were highly welcome and have been implemented into barley breeding schemes. For example, via anther culture, the spring barley cv. 'Henni' (D, 1995), the two-rowed winter barley 'Anthere' (D, 1995), and the six-rowed cultivars 'Uschi' (D, 1997), 'Sarah' (D, 1997), 'Carola', and 'Nelly' (D, 1998) had been released earlier than expected and became widely grown in Europe (E. Laubach, pers. comm.). It is obvious that a substantial time gain can be achieved by the application of this biotechnology step in barley breeding. Consequently, the "haploid breeding method" is widely applied now and has gained great importance for barley breeding as demonstrated by the growing number of DH varieties released (E. Laubach, pers. comm.).

13.6.1.2 Molecular Markers and Marker-Assisted Selection

In addition, the development of molecular markers, which to some extent allow the transfer of selection steps from the phenotypic (field) to the genotypic (laboratory) level, offers new opportunities for a more efficient barley breeding aiming at desired combinations of resistance, yield, and quality. For example, to enhance the resistance against barley yellow dwarf virus (BYDV), Riedel et al. (2011) used DH lines and molecular markers to combine resistance genes Ryd2 and Ryd3 and a quantitative trait locus (QTL) from cv. 'Post' on chromosome 2H. DH lines combining Ryd2 and Ryd2 are reported to show lower virus titer and reduced symptom expression as compared with parental materials. Another example is yellow mosaic-inducing viruses: barley cv. Taihoku A has been described as resistant to yellow mosaic viruses reported in Germany (i.e., BaMMV, BaYMV, BaYMV-2, and BaMMV-Teik). This cultivar carries the BaMMV resistance gene rym13 on chromosome 4H, which may be responsible for the resistance against the whole virus complex. By bulked segregant analysis using simple sequence repeats (SSRs) and amplified fragment length polymorphisms (AFLPs), Humbroich et al. (2010) constructed a map with the closest marker linked to the BaMMV/BaMMV-Teik resistance locus at a distance of 1 cM. These markers are useful tools to introduce resistance to BaMMV-Teik into adapted breeding lines carrying other resistance genes, which are not effective against all BaMMV/BaYMV strains known in Europe. For a long time, powdery mildew has been known as an important pathogen of barley in almost every growing region so that resistance against mildew is of utmost importance. To identify molecular markers, Korell et al. (2008) used a cDNA-AFLP approach to study near-isogenic barley lines differentiated by alleles of the resistance gene Mlg located on chromosome 4H. Based on the identification of a short differential fragment (37 bp), which turned out to be part of a nucleoside diphosphate kinase, a CAPS marker cosegregating with Mlg was developed. Due to its codominance, clear banding pattern, and close linkage, this marker is well suited for marker-assisted selection procedures. While DNA marker analyses gain increasing importance in plant breeding and become more widely adopted in cultivar

development, the capacity for high-throughput analyses at low cost is crucial for its practical application. Automation of the analysis processes is a way to meet these requirements. For this purpose, the company Svalöf Weibull AB, Sweden, has developed a fully automated PCR system. It was evaluated on barley lines and was shown to be capable of analyzing up to 2200 samples per day at costs of EUR 0.24 per analysis for marker-assisted selection and quality control of genetically modified plants (Dayteg et al. 2007).

13.6.1.3 Genome Analysis and GM Barley

Today, sequencing of the barley genome has become a realistic option. Necessary steps to establish and improve genomics tools have been initiated and assembled, coordinated by an international consortium. A suitable reference genome sequence will be an excellent foundation for "selection with markers and advanced recombination technology" (SMART) or marker-assisted breeding, leading to future genomics-based barley improvement. Today, sequencing of the barley genome has become a realistic option. In the foreseeable future, access to highly sophisticated breeding tools with a broad genetic diversity as an absolute basis for gain of selection will probably become the major limiting factor for further breeding progress. Therefore, the conservation, maintenance, and evaluation of plant genetic resources are urgently needed social tasks to build the foundation for future reasonable plant breeding, crop improvement, and entirely successful agriculture. This will be even more the case if expected climate changes will lead to additional requirements of new varieties such as drought or heat resistances. Within the international network of gene banks, for example, the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany, plays a major role not only for barley conservation but also for barley genomics (see Sato and Takeda 2009; Schulte et al. 2009). Genetic transformation is not only necessary for developing new cultivars with specifically modified traits such as improved disease and stress resistance or grain quality.

13.6.2 Molecular Breeding and Genomics in Barley for Biotic Stress Resistance

Barley is characterized by a relatively simple genome structure possessing only a diploid set of chromosomes (seven basic chromosomes of *Triticeae*, H genome). The barley breeding task is being backed up with molecular markers for important traits. For example, in the case of a disease trait, instead of having to go for screening of thousands of lines by inoculating fungus in field, breeders can quickly screen for a DNA tag in the laboratory. The early linkage maps of barley were based on morphological and isozyme markers (Wettstein-Knowles 1992). Later, several barley genetic maps based on a variety of DNA markers (e.g., RFLP, RAPD, AFLPs, STSs, SSRs) were published (Varshney et al. 2005), and detailed information on

most of these maps is available at the GrainGenes website (http://wheat.pw.usda.gov/ggpages/maps.html). Bulked segregant analysis (BSA) has been used by various researchers for mapping resistant gene when a genetic map is not available. Qualitative resistance mechanisms in barley have been extensively studied in terms of genomic location and specificity (Graner and Tekauz 1996).

There is a rich set of genetic resources for barley breeding and genetics including a draft genome sequence, dense genetic maps, extensive mutant collections, TILLING resource, and germplasm collections at the United States Department of Agriculture National Small Grains Collection (USDA-NSGC, USA), the Research Institute for Bioresources (RIB, Japan), and the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Germany). Several genomics databases provide essential tools for breeding and genetics including The Triticeae Toolbox or T3, HarvEST:Barley database (http://harvest.ucr.edu/Barley1.htm), and the Barley Genome Explorer (Barlex; barlex.barleysequence.org). T3 contains barley genetic maps and genotype and phenotype data from germplasm collections, association mapping panels, and breeding lines. T3 also has tools to visualize phenotypic data, conduct clustering analyses, and perform association mapping and genomic prediction. The HarvEST:Barley and Barlex databases provide information on the barley genome sequence and facilitate mapping and cloning projects. A complete barley genome annotation has been published (The International Barley Genome Sequencing Consortium 2012) which will increase the chances to identify candidate resistant genes underlying different diseases.

13.7 Bottlenecks and Prospects for Barley Improvement

13.7.1 Genetic Bottleneck

Since and during domestication, certain selection pressures force the crop plants to change in their genetic base, which "resulted in its fixation and narrowing in cultivated barley in comparison to wild species" (Tanksley and McCouch 1997). Many genes were lost during the process of domestication and modern breeding and warrant introgression of new genes in barley (Kilian et al. 2006). The available genetic resources need to be investigated with modern genetic tools for the much-needed increase in yield potential, as in the past linkage drag, involving undesired linked genes, often hampered their use as parental stocks in plant breeding. Wild gene pools and landraces can be utilized for increasing crop productivity and stress resistance/tolerance under changing environmental conditions (Kilian et al. 2006; Bockelman and Valkoun 2010; Xu et al. 2012). Sub-Saharan Africa and North Africa are the home of barley landraces, which are genetically heterogeneous populations comprising near-homozygous inbred individuals and hybrid segregates generated by a low level of random outcrossing among those individuals in each generation (Nevo 1992). These heterogeneous plant varieties are still being reproduced by farmers as populations and are still subject to both artificial and natural

selection. The traits derived from landraces of barley are principal contributor toward agricultural production, representing over 10 Mha worldwide comprising nine countries with Canada, the USA, and ICARDA in Syria being the major contributors (Altieri 2004). In many developing countries, farmers maintain traditional varieties independently with seed often obtained from relatives, neighbors, or local markets (McGuire 2008). The genetic structure of these landraces may be considered as an evolutionary approach to survival and performance under arid and semi-arid conditions (Schulze 1988) and can hopefully provide a source of alleles for adaptation to climate change.

13.7.2 Pre-breeding and Exploration of Genetic Diversity

A considerable yield advantage of certain landraces over modern varieties in very low rainfall conditions, with little or no use of inputs, has been reported by Ceccarelli and Grando (1996). Barley landraces have developed abundant patterns of variation and would represent a largely untapped reservoir of useful genes for adaptation to biotic and abiotic stresses (Brush 1995) to contribute to the improvement of modern varieties (Veteläinen 1994; Hadjichristodoulou 1995). As an example, for biotic stresses, 19 major genes *(Rph)* for resistance against *Puccinia hordei* have been identified and mapped in barley landraces and wild barley (*H. vulgare* ssp. *spontaneum*) (Weerasena et al. 2004). Generally, in both developing and developed countries, the use of wild crop relatives such as *H. spontaneum* and landraces is not common in barley breeding (Grando et al. 2001). Their breeding programs focus on using newly released varieties and elite germplasm as parents for hybridization. Although most of the recent breeding material is of course originally derived from previous landraces, still, the breeding efforts rely on a relatively narrow gene pool of modern germplasm. Barley landraces are expected to be a source of valuable germplasm for sustainable agriculture in the context of future climate change and provide improved adaptation to local environments (Bellucci et al. 2013). These landrace collections are looked upon as important sources of germplasm with which to enrich modern barley varieties (Tester and Langridge 2010). Exploitation of these landraces in modern crop breeding requires understanding of their phenotypic characteristics, environmental adaptations, and the underpinning genetics, along with their evolutionary relationships. Pre-breeding efforts are essential to be taken up by dedicated basic research programs, as the main breeding programs are focused in variety development, often dependent either on introductions or limited hybridization between improved varieties. The obvious reasons may be the shortage of funds and manpower and priority setting by the concerned institutes. That the incorporation of wild relatives can really be achieved in modern cereal breeding has been successfully proven in bread wheat breeding by CIMMYT (van Ginkel and Ogbonnaya 2007), resulting in several dozens of commercial varieties released to farmers by national programs containing a wild wheat relative as a parent. Their approach can serve as an example for barley breeding.

13.8 Breeding Goals and Projected Progresses

Besides introducing genetic diversity from wild relatives and landraces, a second major focus to improve barley production is on enhancing human and infrastructure capacity of the barley researchers to successfully use modern biotechnological tools. In terms of traits, after yield and yield stability, priority is to be given to disease and pest management through host resistance for sustainable production. Uptake of modern varieties will also be enhanced if distinct varieties will be bred addressing the various agro-ecosystems, including the marginalized, dry, hot environments and the more optimum environments with irrigation potential. Hand-in-hand with genetic improvement, soil fertility and agronomic management of stressed soils, such as due to acidity, salinity, and waterlogging, needs to be undertaken. This requires close cooperation between breeders, agronomists, and also physiologists. Market competitive production will be enhanced if targeted focus is given to improving malting quality to a share of the new varieties. Nutritional security can be improved through micronutrient enrichment by bio-fortification of new varieties, although its priority will depend on other high-priority needs in new varieties. Mechanization emphasis is becoming essential, and need for suitable cultivars to adopt the requirements such as non-brittle spikes becomes an important breeding objective. Depending on where barley research teams are focused, climate change will have different scenarios. In some parts of SSA, increased and more erratic drought is predicted, while in others, rainfall may increase but fall in the form of a limited number of strong outbursts. This requires that research scientists and others along the pathway to provide seed of adapted varieties to farmers need to be very vigilant and focused on monitoring change, so the R&D focus and scope can be adjusted quickly.

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Chapter 14
Finger Millet (*Eleusine coracana* (L.) Gaertn.) Genetics and Breeding for Rapid Genetic Gains

S. Ramesh and T. V. Krishna

14.1 Introduction

Finger millet, commonly known as ragi/African millet, is one of the most ancient crops in the world. It belongs to family Poaceae. It is being cultivated in the arid and semi-arid tropics of Africa and Asia as a food and fodder crop. It is predominantly a rain-fed crop and sometimes it is cultivated under irrigation. It is a low water requirement crop and thus can grow well with a minimum rainfall between 300 and 400 mm. But it stands even up to 1500 mm. In India, it is generally cultivated as a rainy season crop. It is a tall growing herbaceous plant with a tough and robust root growth that enables it to endure and sustain extremely low levels of soil moisture. It supports millions of people living in relatively dry regions of Africa and Asia. Its stover makes an excellent fodder for livestock, especially for draught animals.

14.2 Nomenclature

Its nomenclature evokes much interest as it rings out religio-linguous overtones. Its generic name *Eleusine* is said to have been derived from Greek Goddess of cereals (Chalam and Venkateswarlu 1965). Burkil (1935) opined that it could have been named after Greek town *Eleusi-ne*. As for its specific name, it has been taken from its Ceylonese (Sri Lankan) name Kurukkan. The name finger millet could be obviously from the shape of its earhead, which resembles human palm and the fingers. The name African millet represents its African origin. But 'ragi' which is the common name of finger millet in southern parts of India is colloquial transformation of its Sanskrit name *Rajika*. Incidentally, Sanskrit was

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the widely prevailing lingua franca in ancient India during the period when ragi was acquiring agricultural significance in India.

14.3 Economic Importance

Grains are brick-red in color but some genotypes have white- to peach-colored grains. Grains are tasty, ground into powder (flour), and made into a number of products. Traditionally, foods like porridge (Uji), stiff porridge (Ugali), and local brew Busaa are made for general consumption in Africa. However, in India, the main dishes made are Mudde (dumplings), roti (leaven bread), and porridge (Vaidehi 1986). It is a high-quality energy food and its impact on human health is immense, particularly of working class. Ragi is regarded as a healthy food too. Most important and highly appreciated quality of ragi grain is its low glycemic index and absence of gluten in the grain that makes an ideal food for diabetic patients or those prone to diabetics, a deadly degenerative malady (Vaidehi 1986). Further, due to low protein efficiency ratio value, it is an antidote for obesity. The grain are rich in essential amino acids like methionine and lysine, which are difficult to find generally in grain-based foods (Devos 2005). These qualities might have triggered the awareness about the virtues of ragi and, accordingly, encouraged many countries to plan to include ragi in their diet and cropping systems in recent years. Further, Newman (2005) appreciated its great potential to become a specialty grain for food industry worldwide. Additionally, it is going to be a boon for the people living on subsistence economy in ecologically harsher and imbalanced dry farming zones.

14.4 Origin

Its nativity drew considerable attention and long and lengthy debates and now it is finally settled in favor of Ethiopian Highlands and from where it had moved further down to South Africa and then to India, around 3000–4000 BC (Hilu et al. 1979). But there were no empirical evidences as to when exactly it had entered India. However, Professor Sir J.B. Hutchinson in one of his books, *Essays on Crop Plant Evolution*, published in 1965, had mentioned that a few grains of finger millet along with one of its putative diploid progenitor, viz., *Eleusine indica*, were found in the charred debris in one of the Neolithic sites in Mysore district of Karnataka, India. The approximate age of these grains was reported to have been dated back to 1800 BC. Similar findings in Ethiopia were dated to third millennium BC (Hilu et al. 1979). This unequivocally established the fact that it is of African origin and from where it could have spread over to India later.

Further, for a number of years, it was considered that *Eleusine indica* was a putative parent of *Eleusine coracana*. The fact that *Eleusine indica* was discovered along with *Eleusine coracana* in the debris of excavation dating back to 1800 BC at

Neolithic sites in Mysore district of Karnataka, India, gave an impetus to the claim that it could have been native of India and *Eleusine indica* could have been a probable progenitor of *Eleusine coracana* (Hutchinson 1965). Subsequent cytological and morphological studies, however, elucidated that *Eleusine coracana* could have originated directly from *Eleusine africana* through selection and domestication of a large grain mutant (Hilu and de Wet 1976a, b). This strengthened the speculation and affirmed that it was a native of Africa. It was further confirmed from the findings of archaeological excavation in Ethiopia dating back to three millennium BC (Hilu et al. 1979). It is now an established fact that *Eleusine coracana* has an African origin. *Eleusine indica*, a diploid species with $2n = 18$ chromosomes, is one of the progenitors and the identity of the other donor of the tetraploid *Eleusine coracana* ($2n = 4x = 36$) is still in the realm of guessing.

Yet another evidence in favor of African origin had stemmed from the fact that *E. coracana*, a tetraploid with $2n = 36$ chromosomes, generally cultivated in India, had cytological and morphological features, which suggested that it was genetically conspecific to another tetraploid species *Eleusine africana* Kennedy O'Byrne with $2n = 36$ chromosomes, mostly found in Africa (Chennaveeriah and Hiremath 1974; Hilu and de Wet 1976a, b). Additionally, they observed that gene flow occurred freely between these two species. From this, they deduced that *Eleusine coracana* might have originated directly from *Eleusine africana*, possibly as a mutant, and subsequently selected for its larger grain size and then cultivated as finger millet, which are of two types: (1) African highland race, and (2) Afro-Asiatic lowland race. Hilu and de Wet (1976b) proposed that the African highland race was derived from *E. africana* and this then gave rise to African lowland race, which was then introduced to India. However, more authentic or clinching evidence that it was from East African origin came from the archaeological finding of finger millet in Ethiopia by Hilu et al. (1979), in which the finger millet-like grains were found in the debris, which were dated back to 3000 BC. Moreover, evidence for more ancient nature of *E. coracana* in India was derived from the archaeological finding of *Eleusine coracana* together with *Eleusine indica* near Halaguru, Mysore district of Karnataka, India, which were dated back to 1800 BC (Hutchinson 1965). Now both the races are designated as *Eleusine coracana*, subspecies *coracana*, and *Eleusine coracana*, subspecies *africana* (Acheampong et al. 1974–84).

14.5 Distribution

By virtue of its outstanding food and agricultural characteristics together with excellent nutraceutical properties, finger millet has spread to many countries in the world. Presently, it is found in almost all the eastern and southern African nations such as Kenya, Uganda, Zimbabwe, Tanzania, Rwanda, Zaire, and South Africa, besides Ethiopia. In Asia, India is the major ragi-growing country in the world. Besides, Nepal is slowly emerging as an important ragi-growing country. Malaysia, Indonesia, Japan, and China also figure in the ragi map of world. Recent reports

have alluded to the fact that it has made a beginning in the United States of America too. But presently, the grain is mostly used as bird feed. In India, its distribution is practically all over the country. Even in temperate Himalayas, it is being grown up to the elevation of 2300 m. But its concentration is in the states of Karnataka, Andhra Pradesh, Tamil Nadu, Maharashtra, Odisha, Jharkhand, and so on. Karnataka maintains the largest acreage and is a leading state in production.

14.6 Botany

Eleusine is a small genus comprising only 11 species. The chromosome numbers in the genus range from 18 to 45. Since there has been no reported species with less than 18 diploid chromosomes, it was proposed that its basic chromosome number could be $x = 9$. On this premise, 11 species in the genus are classified into diploids ($2n = 18$), tetraploids ($2n = 4x = 36$), and pentaploids ($2n = 5x = 45$). The diploids are *Eleusine obligostachya* Lam, *Eleusine coracana* (*Linn*), and *Eleusine verticillata* Roxb. The only pentaploid with 45 chromosomes is *Eleusine flagellifera.* It is apparent now that the species differentiation in the genus is based on the multiples of the basic chromosome number $x = 9$. Thus, there are altogether six diploids, four tetraploids, and one pentaploid.

Of all these species, *Eleusine coracana* is the only one that acquired agricultural significance both in Africa and India. It is a tall, annually growing herbaceous with a height of a meter or so, ending with an inflorescence called umbel or panicle, having finger-like spikes—and hence also called finger millet. Its growth habit is decumbent/erect/prostrate. Its stem is compressed with nodes, and thickness varies from 0.4 to 10.3 mm. It tillers profusely and they arise from the base of the plant (collar region). They vary from one to ten per plant, but not all of them are productive. Number of leaves varies according to height of the plant. They are long and linear with a prominent midrib, which tapers into an acuminate tip. They are generally glabrous and often found with ciliate margins. They are attached to the stem through a sheath, which firmly clasps the stem right from the internode. In addition to normal leaves, there is a flag leaf, arising from the lost internode, just below the thumb (odd) finger, which is situated a few millimeters or a centimeter down from the base of the panicle or earhead. The main stem and tillers end up in earhead, which consist of finger-like spikes. The earhead is borne on the peduncle whose length varies from 11.5 to 59.5 mm and width ranging from 11.9 to 15.56 mm. Number of fingers varies from four to five and sometimes even more. Little below the main fingers, generally, there is another finger, which is known as thumb finger. The shape and size of earhead vary. They are small, intermediate, and large. They are open or fist-like, the later due to incurved and compact fingers. Fingers consist of spikelets and are crowded into two overlapping rows on either side of the rachis. Each spikelet contains four to five flowers (florets). Flowers are bigger at the base than at the median line. There is a keel with short, stiff hairs. The florets are hermaphrodite, except the terminal florets. There

are three stamens with short anthers and long filaments; the lobes of anthers dehisce longitudinally; gynoecium is bicarpellary, unilocular, and with superior ovary. There are two broad and truncated lodicules that are present at the base of the ovary. The obovate ovary possesses a distinct style and plumose stigma. The florets open in basipetal successions in the spikelets (Umashankar and Setty 1977). Umashankar and Setty (1977) and Dodake and Dhonukshe (1998) observed that anthers dehisce around 3.00 am and pollination takes place immediately thereafter; though self-pollination is normally expected, some amount of out-crossing is also observed. However, the latter is genotype-specific (Fakrudin et al. 1998).

Seed is an achene. The seeds vary in color and shape. Seeds are covered by the glumes, but there are variations: exposed, partially covered, and completely covered. Partially covering categories are more frequent. There are differences in the grain color: white, light brown, copper brown, and purple brown. Copper brown types are more frequent (brick-red). Grains differ in shapes: round, reniform, and ovoid. Round-shaped grains are more frequent. Grain surface is either smooth or wrinkled; the former is more predominant.

14.7 Cytogenetics

*Eleusine coracana*hasattracted a great deal of attention, both in terms of origin and evolutionary points of view. There was a general perception that *Eleusine coracana* was a tetraploid form of diploid *Eleusine indica*, a grassy weed, ubiquitous in its distribution, endowed with diploid chromosome number of $2n = 18$ (Krishnaswamy 1951; Mehra 1963). Kempanna et al. (1976), however, carried out detailed karyomorphological studies of both the species, that is, *E. coracana* and *E. indica*, and compared them. The chromosomes of *E. coracana* (Table 14.1) were longer than those of *E. indica* (Table 14.2). Further, while *E. coracana* consisted of two satellite chromosomes (Fig. 14.1), *E. indica* consisted of only one satellite chromosome (Fig. 14.2). However, in both the species the satellite chromosomes were longer than those of others.

Further, Nayar et al. (1978) investigated karyotype of five diploid species (*Eleusine trystachya*; *Eleusine jaegeri*; *Eleusine floccifolia*; *Eleusine boronensis*; *E. indica*) and two tetraploid species (*E. africana*; *E. coracana*). While the three diploid species (*E. trystachya*; *E. jaegeri*; *E. floccifolia*) had two satellite pairs each, *E. boronensis* had two satellite pairs. Among tetraploids, *E. coracana* had two satellite pairs while *E. africana* had six satellite chromosomes. One of the satellite pair was longest in *E. coracana.* Further, karyotypes of four (*E. trystachya*; *E. jaegeri*; *E. floccifolia*; *E. indica*) out of five diploids were asymmetrical whereas those of *E. boronensis* were symmetrical. Among the five diploids, the total chromatin length was shortest in *E. indica*; it was longest in *E. trystachya*. Among the two tetraploids, the total chromatin length was shortest in *E. africana* (Table 14.3). All the diploids, except *E. indica*, were closely related to each other.

Table 14.1 Karyotype of *Eleusine coracana*

Chromosome number	Length (μm)	Arm ratio (μm)
1	3.090 ± 0.0203 satellite	1.1074 ± 0.0054
2	2.975 ± 0.0465 satellite	1.1071 ± 0.0063
3	2.890 ± 0.0663 special chromosome	1.1053 ± 0.0017
4	2.695 ± 0.0298	1.1026 ± 0.0017
5	2.630 ± 0.0289	1.1007 ± 0.0012
6	2.530 ± 0.0289	1.1083 ± 0.0014
7	2.489 ± 0.0416	1.1049 ± 0.0012
8	2.435 ± 0.0215	1.1029 ± 0.0010
9	2.390 ± 0.0340	1.1016 ± 0.0013
10	2.350 ± 0.0419	1.1061 ± 0.0013
11	2.290 ± 0.0419	1.1044 ± 0.0013
12	2.215 ± 0.0379	1.1036 ± 0.0014
13	2.200 ± 0.0368	1.1037 ± 0.0013
14	2.110 ± 0.0189	1.1089 ± 0.0009
15	2.050 ± 0.0275	1.1085 ± 0.0016
16	2.005 ± 0.0025	1.1005 ± 0.0005
17	2.000 ± 0.0000	1.000 ± 0.0000
18	1.900 ± 0.0000	1.111 ± 0.0000

Source: Kempanna et al. (1976)

Table 14.2 Karyotype of *Eleusine indica*

Chromosome number	Length (μm)	Arm ratio (μm)
1	2.85 ± 0.0055 satellite	1.1025 ± 0.0008
2	2.189 ± 0.006 special chromosome	1.3222 ± 0.0072
3	2.19 ± 0.0068 special chromosome	1.1046 ± 0.001
4	2.13 ± 0.0067	1.1057 ± 0.0013
5	2.07 ± 0.0057	1.1047 ± 0.0014
6	2.03 ± 0.0055	1.1061 ± 0.0081
7	1.96 ± 0.0065	1.1057 ± 0.0014
8	1.885 ± 0.0055	1.1060 ± 0.0015
9	1.73 ± 1.1059	1.1059 ± 0.0015

14.8 Genetic Resources

Genetic resources are the wealth/treasure for continuous genetic improvement of economically important crops to cater to the needs of present and future generations. Considering the importance of finger millet for food security, especially in production systems with frequent drought spells, concerted efforts have led to collection and conservation of a large number of germplasm accessions at different institutes/universities (Table 14.4).

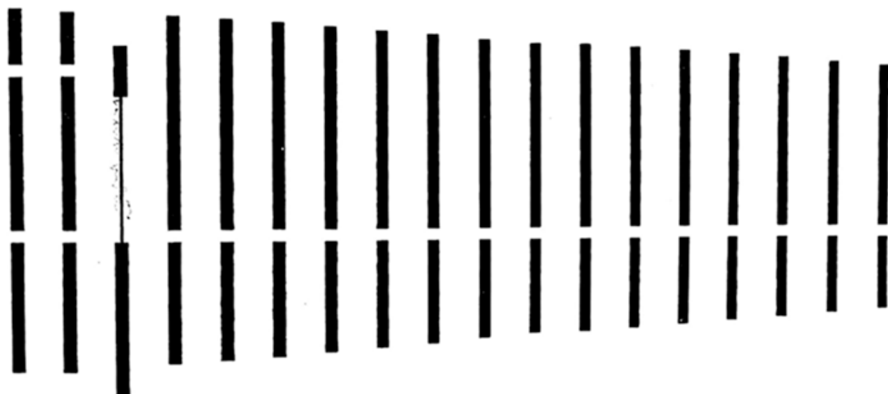
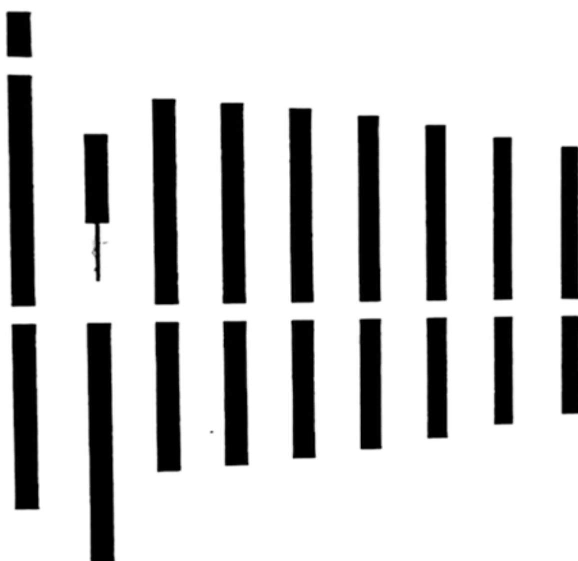


Fig. 14.1 Ideogram showing the average length and arm ratio of the 18 pairs of chromosomes in *Eleusine indica*

Fig. 14.2 Ideogram showing the length and arm ratio of nine pairs of *Eleusine indica*



The largest collection of finger millet genetic resources is held in institutes located in India, for example, National Bureau of Plant Genetic Resources (NBPGR), New Delhi, International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, and University of Agricultural Sciences (UAS), Bengaluru. Several researchers have attempted to evaluate and characterize fairly large numbers of germplasm accessions. For example, Kempanna and Tirumalachar (1968), Mallanna et al. (1978), and Bhaskariah and Mallanna (1997) evaluated 619, 925, and 1064 *E. coracana* accessions, respectively, in various years. They reported considerable

Table 14.3 Comparison of karyotypes of five diploids and two tetraploid species of *Eleusine*

Sl. no.	Name of the species	Ploidy level	Type of karyotype	Length of chromosomal range (μm)	Total chromatin length (μm)
1	*Eleusine trystachya*	Diploid	5 M + 4 SM	2.40–3.40	51.12
2	*Eleusine jaegeri*		4 M + 5 SM	1.92–3.30	46.74
3	*Eleusine floccifolia*		4 M + 5 SM	2.25–2.76	44.88
4	*Eleusine boronensis*		3 M + 6 SM	2.32–3.60	48.24
5	*Eleusine indica*		2 M + 7 SM	1.40–2.36	29.12
6	*Eleusine coracana*	Tetraploid	12 M + 6 SM	1.80–3.48	81.00
7	*Eleusine africana*		8 M + 10 SM	0.88–2.84	68.00

Source: Nayar et al. (1978)
M median centric, *SM* sub-median centric

Table 14.4 Significant germplasm collections of finger millet

Sl. no.	Institution	Headquarters	Number of accessions
1	National Bureau of Plant Genetic Resources (NBPGR)	New Delhi, India	9522
2	International Crops Research Institute for Semi-Arid Tropics (ICRISAT)	Patancheru, India	6804
3	All India Coordinated Minor Millet Project (AICMMP)	University of Agricultural Sciences (UAS) Bengaluru, India	6257
4	Kenya Agricultural Research Institute (KARI)	Muguga, Kenya	2875
5	Institute of Biodiversity Conservation (IBC)	Addis Ababa, Ethiopia	2156
6	USDA Agricultural Research Service (USDA-ARS)	Griffin, USA	1452
7	Serere Agricultural and Animal Production Research Institute (SAARI)	Soroti, Uganda	1231
8	SADC Plant Genetic Resource Centre	Lusaka, Zambia	1037
9	Central Plant Breeding and Biotechnology Division, Nepal Agricultural Research Council (CPBBD)	Kathmandu, Nepal	869
10	National Center for Genetic Resources Preservation	Fort Collins, USA	702
11	National Institute of Agrobiological Sciences (NIAS)	Kannondai, Japan	565
12	Mt. Makulu Central Research Station	Chilanga, Zambia	390
13	Institute of Crop Germplasm Resources, Chinese Academy of Agricultural Sciences (ICGR-CAAS)	Beijing, China	300

Source: Goron and Raizada (2015); Dwivedi et al. (2012)

variability in both qualitative and quantitative traits. There have been several other numerous efforts to evaluate the germplasm accessions, but with limited numbers. In an effort to broaden the genetic base of finger millet cultivars, Gowda and Sheriff (1986) generated variability from inter-species (*E. coracana* × *E. africana*) crosses and reported significant variability in most of the economically important traits. The natural variability that existed among the accessions has been exploited to identify high-yielding cultivars as a short-term strategy to cater to immediate needs of the farmers.

Considering that the genetic resources held at NBPGR, New Delhi, ICRISAT, Patancheru, and UAS, Bengaluru, are unwieldy for precise characterization and evaluation and that there is possibility of occurrence of duplicates due to repeated sampling of same accession and/or assigning different names/identity to the same accession, core sets consisting of varying numbers of accessions have been developed. A team of scientists under the leadership of Dr. A. Seetharam, Former Project Coordinator of All India Coordinated Small Millets Improvement Project (AICSMIP), University of Agricultural Sciences (UAS), Bengaluru, India, developed a core set of 551 accessions based on phenotypic evaluation of global collection of 5669 accessions during 1996–2005 in India at four locations, namely Almora (in the Himalayas), Ranchi (North India), and Vizianagaram and Bengaluru (in the Deccan plateau region). This core set comprised the accessions originating from ten countries representing Africa (primary center of diversity), the Indian subcontinent (secondary center of diversity), and others (Table 14.5; Seetharam et al. 2005).

The core set of accessions have been deposited at the National Active Germplasm Site, AICSMIP, Bengaluru, India. Subsequently, Upadhyaya et al. (2006) also developed a core set of 622 accessions from a total of 5940 accessions held at International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India, based on 14 quantitative traits. Newman (2005) of Plant Genetic

Table 14.5 Country representation of core set of finger millet germplasm accessions

Country	No. of accessions
India	379
Malawi	55
Kenya	48
Uganda	24
Zambia	12
Zimbabwe	07
Tanzania	04
Sri Lanka	03
Japan	03
Others	14

Resource Conservation Unit at Griffin, USA, developed yet another core set of 80 accessions, representing over 90% variability in the base collection of 700 accessions. The core set is suggested for evaluation across target production environments and years to identify widely/specifically adapted and stable accessions to foster enhanced access and use of finger millet germplasm in cultivar development. The core sets are considered as first-look sources of genetic resources for use in crop improvement programs. The availability of core sets is expected to result in enhanced utilization of genetic resources in crop breeding programs, which is the key to develop cultivars with broad genetic base, which contribute to sustainable production of finger millet.

14.9 Genetics

14.9.1 Qualitative Traits

Several researchers have reported the number and mode of action of genes controlling easily observable/assayable and highly heritable traits such as stem, earhead, and grain color. These traits are controlled by one to three genes (Table 14.6).

These traits could be used as diagnostic markers of germplasm accessions for maintaining their identity and purity. They help minimize duplication and avoid mistakes in labeling the germplasm accessions and thereby enable their easy retrieval from the collection. They can also be used in detecting true hybrids considering that developing hybrids in finger millet is tedious owing to tiny florets. They

Table 14.6 Genetics of qualitative traits in finger millet

Trait	Number of genes	Mode of action	Reference
Plant pigmentation: purple/green	One	Purple is dominant over green	Ravikumar and Seetharam (1990)
Length of rachis	Two dominant genes: E_1 and E_2	Complementary epistasis	Vijayaraghavan and Warier (1949)
Earhead color (purple vs. green)	One with two alleles P and p	Purple dominant over green; P > p	Ayyangar and Warier (1933)
Glume cover: complete/partial	Three genes	Complementary epistasis	Ayyangar and Warier (1931)
Grain color: purple/green; brown/green	Two genes	Purple and brown colors are dominant over green; duplicate dominant	Shanthakumar and Gowda (1998)
Genetic male sterility (GMS)	One	Fertility is dominant over sterility	Gupta et al. (1997)

are found useful in conducting Distinctness (D), Uniformity (U), and Stability (S) test, a mandatory requirement for protecting varieties under Protection of Plant Varieties and Farmers' Rights (PPV&FR) Act of India and such other similar Acts that are in vogue in other countries.

14.9.2 Quantitative Traits

Owing to the difficulty in effecting crosses, attempts to investigate genetics of quantitative traits are limited. Review of a few such studies (Sumathi et al. 2005; Gurunathan et al. 2006; Gupta and Kumar 2009; Shailaja et al. 2009) has indicated that most of the economically important traits such as grain yield and its components are controlled by genes with predominantly dominant mode of action.

14.10 Breeding

Major efforts to breed finger millet were concentrated in India. Breeding finger millet in Africa is rather limited. Breeding is predominantly focused on improving grain yield and its components and resistance to blast disease.

14.10.1 Breeding for Productivity Per Se Traits in India

Tyagi and Rawat (1989) bred two varieties, Pant Mandu 3 and PES 110, in Uttarkhand state of India in the Himalayas. Both were tolerant to leaf, finger, and neck blasts. The former matured in 95 days. It was 80–85 cm tall with compact and curved spikes and the seed was light brown in color. The variety PES 110 matured in 115–120 days, and had medium-sized top. First-ever attempt on breeding finger millet was initiated by Dr. Leslie C. Coleman in the then Mysore state (now Karnataka) in India by about 1900 AD. He made several collections of finger millet accessions from Mysore and Madras province (now Tamil Nadu) in South India and quantified variability in farmer-preferred traits at Hebbal, Bengaluru, India. From this initial attempt, he identified seven different types based on earhead shape and color. A few of them produced long and open earheads with green color, while others produced small, closed, compact purple-pigmented earheads. This study led him to isolate a high-yielding genotype from locally cultivated, nondescript variety "Madayyanagiri." It was a tall, purple-pigmented variety having a better yield structure than its parental stock and was found suitable for dryland cultivation. It was released in the state of Mysore in 1922 (Coleman 1922). It had remained as a variety

for a pretty long time. At the same time, Tamil Nadu developed and released two varieties CO 1 and CO 10, which combined good yields with better protein content.

These were all pure-line selections from the landraces. Hybridization was difficult with the crop as the florets in the spikes were small and embedded in the densely crowded spikelets. To overcome this difficulty, Ramaswamy et al. (1994) suggested "contact method of hybridization." In this method, earheads of the two selected varieties are tied together and covered with grease-proof paper bags. Subsequently, seeds are collected from the earheads after they mature, seedlings from such seeds are raised, and plants harboring traits from both the parents are selected as true hybrids. A few popular varieties like Purna, Annapurna, and Cauvery were identified through pedigree selection from segregating populations derived from crosses developed using contact method of hybridization at Mandya Centre in India. While the variety "Cauvery" was suitable for dryland ecosystem, "Purna" and "Annapurna" were suitable for both dry and irrigated ecosystems. Following the "contact method of hybridization," the first-ever Indo-African variety HR 374 was developed, which was a cross between EC 4840 and HES 927 (now IE 927). This was a very high yielder. It was released for cultivation in Karnataka in 1997. Another variety, HR 911, a cross between UAS 1 and IE 927, was adaptable to both rainy (June–October/November) and summer (February–May) seasons and was released for cultivation during 1985 (Gowda and Sheriff 1986). These "Indaf" series varieties had a very high yield potential and replaced almost all the earlier released varieties. These varieties brought a paradigm shift in ragi production scenario in India.

At Mandya Centre, a regional research station of UAS, Bengaluru, Lakshmanaiah in 1970s and 1980s extensively used exotic accessions, IE 927, IE 929, and IE 980 R, and developed a series of Indaf (Indian × African accessions) varieties (Indaf 1–9) with high-yielding ability (Fig. 14.3). Indaf 1 was more suitable for kharif season and Indaf 5 for summer season, and Indaf 9 was good for late rainy season. Subsequently, Indaf 1 and Indaf 5 were replaced by a better variety Indaf 8. Indaf 7 was released for postrainy season. Gowda and Sheriff (1986) developed another variety from a cross between PR 209 and IE 927, which had high yield potential in respect of both grain and stover, besides tolerance to drought and lodging. It was released for transitional zone in southern Karnataka. To improve grain quality with high protein, a variety "Hamsa" was crossed with brown-seeded variety IE 927 and developed Indaf 11 with better protein quality. In extensive field trials for 3 years during 1981 through 1984, three new varieties, HR 911, Indaf 8, and Indaf 5, were developed. Gowda et al. (1999) developed a variety HR 391, which was suitable for rain-fed cultivation in dry belt of southern Karnataka. It matured in 118–120 days. Gowda et al. (1999) developed a dual-purpose variety MR 2 for Southern Transition belt of Karnataka. It was a hybrid derivative of PR 202 × IE 927, developed at Mandya Centre. It had superior grain yield together with tolerance to drought and lodging. Of late, germplasm unit (GPU)series of varieties (Fig. 14.4) such as GPU 28, GPU 45, GPU 48, GPU 66, and GPU 67 has been developed and released for commercial production. Sundareshan and Prasad (1983) reported a variety CO 12, which was a selection

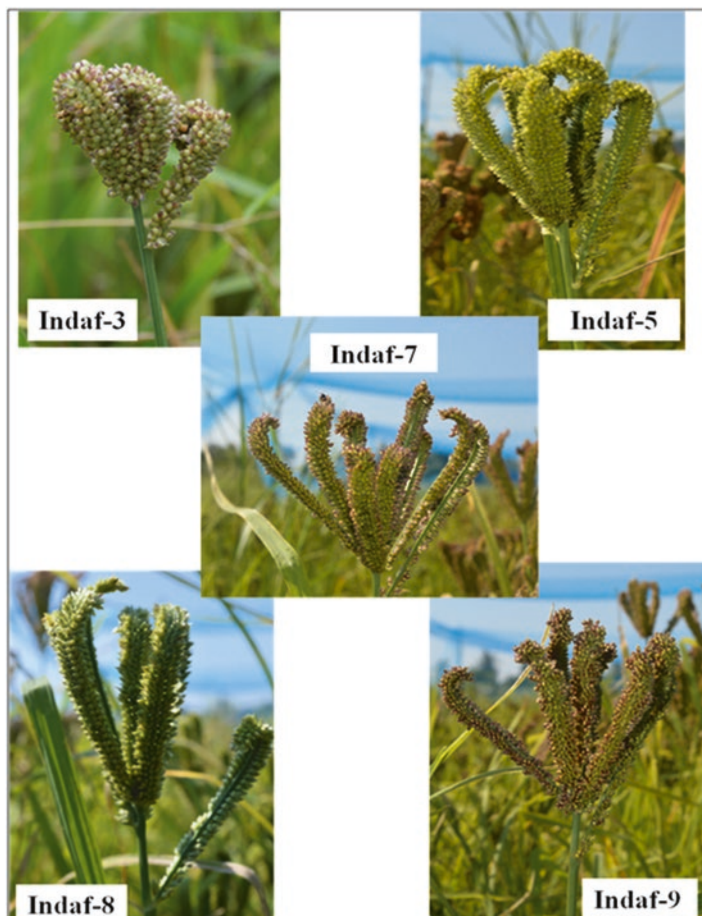


Fig. 14.3 Photographs of panicles of Indaf series finger millet varieties derived from Indian × African germplasm crosses

having medium duration and matured in 85–100 days. It was suitable for Tamil Nadu in India. It was developed at Directorate of Wheat Research at Karnal in Haryana in North-West India. A comprehensive list of varieties released for commercial production in India is presented in Table 14.7.

14.10.2 Breeding Finger Millet in Africa

Gupta et al. (1989–90) identified a high-yielding genotype from a germplasm accession P 1462703 and registered in Zimbabwe in 1986. It matured in 87 days and had a medium grain size with good malting ability. Subsequently, Mnyenyembe (1990)

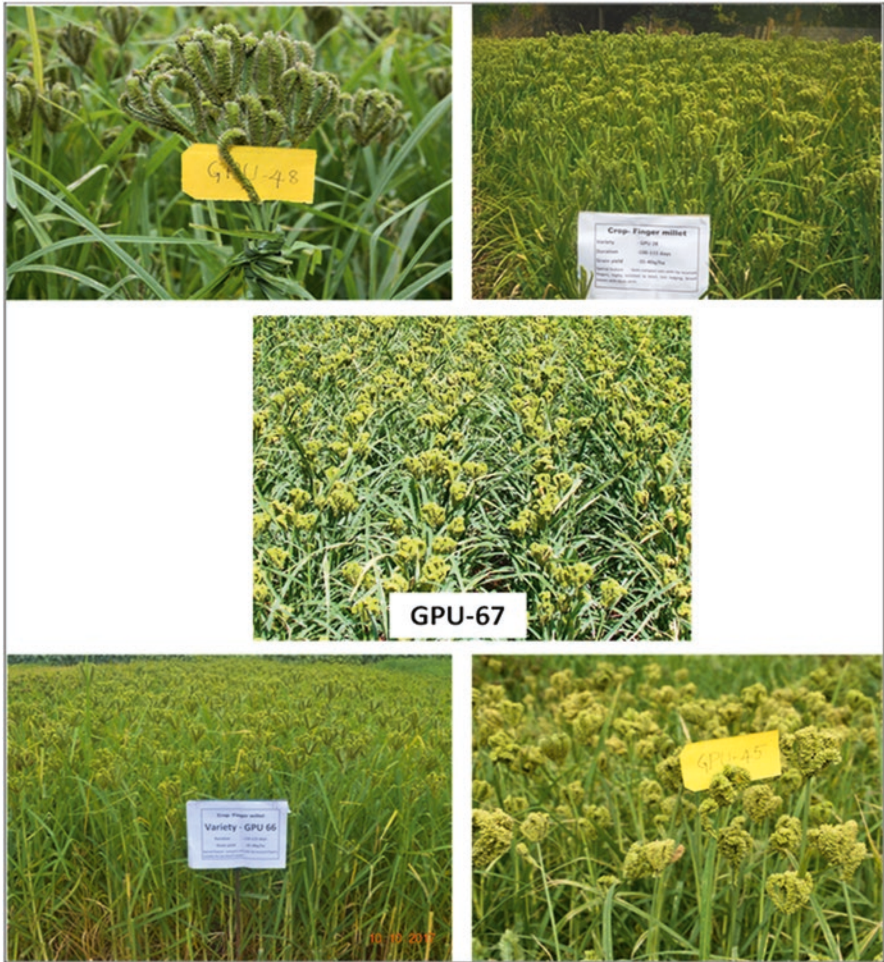


Fig. 14.4 Photographs of germplasm unit (GPU) series of finger millet varieties

identified an early maturing variety "FMVI" from a landrace with good brewing ability in Zimbabwe. It matured in 90–115 days and was blast and drought resistant. Mnyenyembe (1990) tested 25 selections at seven locations in Malawi during 1974–1975 and identified a high-yielding selection. Subsequently, he identified two more high-yielding selections. Based on advanced early maturing varietal trials involving 25 selections at four locations in Zambia, Gupta et al. (1989–90) identified a highest-yielding variety "SDRM 3" that accounted for an increase of 42% over the check variety.

Table 14.7 Finger millet varieties released in India

Sl. no.	Name of variety	Year of release	Pedigree	Institution developed
1	Chhattisgarh Ragi-2 (BR-36)	2018	PR-202 × GE-669	Indira Gandhi Krishi Vishwavidyalaya (IGKVV), Jagdalpur
2	DHFM-78-3	2018	GE 1219 × Indaf 8	University of Agricultural Sciences (UAS), Dharwad
3	Dapoli-2 (SCN-6)	2017	Soma-clone of Dapoli-1	Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth (BSKKV), Dapoli
4	CO 15	2017	CO 11 × PR 202	Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu
5	GNN-7	2017	Pure-line selection	Navsari Agricultural University (NAU), Gujarat
6	VL-379	2017	GE-440 × VL-149	Indian Council of Agricultural Research - Vivekananda Parvatiya Krishi Anusandhan Sansthan (ICAR-VPKAS), Almora
7	KMR 340	2016	OUAT-2 × WRT-4	UAS, Bengaluru
8	VL 376	2016	GE 4172 × VL Ragi 149	ICAR-VPKAS, Almora
9	GNN-6	2016	Selection from local germplasm WN-259	NAU, Waghai
10	GN-5	2016	Selection from local germplasm WWN-20	NAU, Waghai
11	VL Mandua-348	2016	VL Ragi 146 × VL Ragi 149	ICAR-VPKAS, Almora
12	VL 352	2012	VR 708 × VL 149	VPKAS, Almora, Uttarakhand
13	Indira Ragi-1	2012	HR 911 × GE 669	Agricultural Research Station (ARS), Jagdalpur, Chhattisgarh
14	PPR 2700 (Vakula)	2012	KM 55 × U 22/B	ARS, Perumallapalli, AP
15	VR 396 (Hima)	2012	IE 2695 × PR 202	ARS, Vizianagaram, AP
16	KMR 204	2012	GPU 26 × GE 1409	Vishweshwaraiah Canal (VC) Farm, Mandya, Karnataka
17	OEB 532	2012	GPU 26 × L5	Orissa University of Agriculture and Technology (OUAT), Berhampur, Odisha
18	OEB 526	2012	SDFM 30 × PE244	OUAT, Odisha
19	KOPN 235 (Phule Nachni)	2011	Pure-line selection	Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri
20	VL 347	2010	VR 708 × VL 149	VPKAS, Almora, Uttarakhand
21	PRM 2	2010	Pure-line selection	GB Pant University of Agriculture and Technology (GBPUAT), Uttarakhand
22	GPU 67	2009	Selection from GE 5331	UAS, Bengaluru

(continued)

Table 14.7 (continued)

Sl. no.	Name of variety	Year of release	Pedigree	Institution developed
23	KMR 301 (Gowri)	2009	MR 1 × GE 1409	UAS, Bengaluru
24	GPU 66	2009	PR 202 × GPU 28	UAS, Bengaluru
25	VR 847 (Srichaitanya)	2009	GPU 26 × L5	ARS, Vizianagaram, Andhra Pradesh
26	GN 5	2009	Pure-line selection	NAU, Waghai, Gujarat
27	ML 365	2008	IE 1012 × Indaf 5	UAS, Bengaluru
28	Paiyur 2	2008	VL 145 × Selection 10	TNAU, Coimbatore
29	VR 762 (Bharathi)	2006	Pure-line selection from VMEC 134	ARS, Vizianagaram, AP
30	VL 324	2006	VL 162 × IE 3808	VPKAS, Almora, Uttarakhand
31	PRM 1	2006	Pure-line selection Ekeshwar local	GBPAUT, Ranichauri, Uttarakhand
32	VL 332	2006	VL 127 × IE 1213824	VPKAS, Almora, Uttarakhand
33	GN 4	2006	Selection from WN228	NAU, Waghai, Gujarat
34	GPU 48 (Ratna)	2005	GPU 26 × L 5	PC Unit, UAS, GKVK, Bengaluru
35	DHRS 1	2005	Pure-line selection	ARS, Hanumanamatti, Karnataka
36	VL 315	2004	SDFM 69 × VL 231	VPKAS, Almora, Uttarakhand
37	Co 14 (TNAU 946)	2004	Malawi 1305 × Co 13	TNAU, Coimbatore
38	MR 6 (Divya)	2004	African white × ROH2	UAS, Bengaluru
39	GPU 45	2001	GPU 26 × L5	UAS, Bengaluru
40	OEB 10 (Chilika)	2001	GE 68 × GE 156	OUAT, Berhampur, Odisha
41	L 5	1999	Malawi × Indaf 9	ARS, Nagenahalli, Karnataka
42	OUAT 2 (Surya)	1999	Mutant of C09	OUAT, Berhampur
43	BM 9–1 (Bhairabi)	1999	Mutant of Budha Mandia	OUAT, Berhampur
44	PR 230 (Maruthi)	1998	Pure-line selection	Regional Agricultural Research Station (RARS), Andhra Pradesh
45	VR 708	1998	Pure-line selection-VNEC36	ARS, Vizianagaram, Andhra Pradesh
46	GPU 28	1998	Indaf 5 × (Indaf 9 × IE1012)	UAS, Bengaluru
47	GPU 26	1997	Indaf 5 × Advanced line derived from Indaf 9 × IE1012	UAS, Bengaluru
48	BM 11–1 (Rushikulya)	1996	Mutant of Buddha Mandia	OUAT, Berhampur
49	PR 2614 (Saptagiri)	1995	MR 1 × Kalyani	ARS, Perumallapalli
50	BM 2 (Birsa Marua)	1995	Pure-line selection	Birsa Agricultural University (BAU), Ranchi, Jharkhand

(continued)

Table 14.7 (continued)

Sl. no.	Name of variety	Year of release	Pedigree	Institution developed
51	VL 146	1995	VL 201 × IE 882	VPKAS, Almora, Uttarakhand
52	MR 2 (Akshaya)	1994	Indaf 5 × PR 202	UAS, Bengaluru
53	KM 65	1994	Pure-line selection	Chandra Shekhar Azad University of Agriculture & Technology (CSAUA&T), Kanpur
54	DAPOLI 1	1994	Selection from Mutant no. 50/1	BSKKV, Dapoli, Maharashtra
55	PPR 2350 (Padmavathi)	1993	Pure-line selection	ARS, Perumallapalli
56	GN 3	1993	KM13 × GN 2	Gujarat Agricultural University (GAU), Gujarat
57	A 404	1993	Introduction from AP	Birsa Agricultural University (BAU), Ranchi
58	PR 1158–9 Goutami)	1992	Godavari × U22	ARS, Vizianagaram
59	VR 520 (Suraj)	1992	Pure-line selection from VN2507/19	ARS, Vizianagaram
60	INDAF 15	1991	IE 67 × IE 927	UAS, Bengaluru
61	VL 149	1991	VL 204 × IE 882	VPKAS, Almora, Uttarakhand
62	MR 1	1990	Hamsa × IE 927	UAS, Bengaluru
63	VL 124	1989	Pure-line selection	VPKAS, Almora, Uttarakhand
64	TRY 1 (SSRC 247)	1989	Pure-line selection HR374	TNAU, Coimbatore
65	RAU 8	1989	BR 407 × Ranchi local	Dr. Rajendra Prasad Central Agriculture University, formerly Rajendra Agricultural University (RAU), Dholi, Bihar
66	KM 13	1989	Pure-line selection	CSAUA&T, Kanpur
67	PES 400 (Pant Mandua 3)	1989	Pure-line selection	GBPUAT, Pantnagar
68	Co 13 (TNAU 294)	1989	Co7 × TAH 107	TNAU, Coimbatore,
69	HR 911 (KBR 1)	1985	UAS 1 × IE 927	UAS, Bengaluru
70	INDAF 9 (Chitta)	1985	K1 × IE 980R	UAS, Bengaluru
71	VL 204	1985	Pure-line selection	VPKAS Almora, Uttarakhand
72	Simhadri	1985	Pure-line selection from VN311	ARS, Vizianagaram
73	PR 1044 (Ratnagiri)	1985	Pure-line selection local Mettachody variety/PM 629	ARS, Peddapuram
74	PES 110	1985	Pure-line selection	GBPUAT, Pantnagar

(continued)

Table 14.7 (continued)

Sl. no.	Name of variety	Year of release	Pedigree	Institution developed
75	Co 12	1985	Pure-line selection from PR722	TNAU, Coimbatore
76	NILCHAL (B-4-10-56)	1985	Mutant of IE 642	OUAT, Bhubaneswar
77	PAIYUR 1	1985	Pure-line selection from PR 722	TNAU, Coimbatore
78	BM 1 (IE 723)	1985	Pure-line selection	BAU, Ranchi
79	K 7	1982	GO 8 × K2	TNAU, Coimbatore
80	GN 2	1982	Pure-line selection from Gujarat local	GAU, Gujarat
81	INDAF 8	1982	Ullubele × IE 929	UAS, Bengaluru
82	K 6	1982	Natural Mutant from local	TNAU, Coimbatore
83	K5	1982	Sarada × EC 158	TNAU, Coimbatore
84	Co 11 (EC 4849)	1982	Pure-line selection from MS2584	TNAU, Coimbatore
85	INDAF 7 (Hasta)	1981	Annapurna × IE 927	UAS, Bengaluru
86	NIRMAL	1980	Pure-line selection from genetic collection from Nepal	CSAUA&T, Kanpur
87	VL 101	1978	Pure-line selection from IE 524	VPKAS, Almora, Uttarakhand
88	INDAF 5	1977	Kaveri × IE 929	UAS, Bengaluru
89	INDAF 3	1976	Kaveri × IE 927	UAS, Bengaluru
90	PR 202 (Godavari)	1976	Pure-line selection from Mettachudyragi of Araku variety	ARS, Peddapuram, Andhra Pradesh
91	INDAF 1	1976	Ullubele × IE 929	UAS, Bengaluru
92	Co 10	1976	Pure-line selection Maruaragi	TNAU, Coimbatore
93	HPB 7–6	1976	Hamsa × Poorna	UAS, Bengaluru
94	GN 1 (Gujarat Nagli 1)	1976	Pure-line selection from local selection of Dangs	GAU, Gujarat
95	HR 374	1975	EC 4840 × IE 927	UAS, Bengaluru
96	Kalyani	1972	Pure-line selection from CR 652	ARS, Perumallapalli
97	Shakti	1972	R0013 × H 22	UAS, Bengaluru
98	Dibya Sinha	1971	Mutant of AKP 7	Centre for Pulses Research (CPR), Berhampur
99	AKP 7 (Sarada)	1971	Pure-line selection	RARS, Anakapalli, AP
100	CO 9	1970	EC 4336 × PLR 1	TNAU, Coimbatore
101	Hamsa	1967	Pure-line selection	UAS, Bengaluru

(continued)

Table 14.7 (continued)

Sl. no.	Name of variety	Year of release	Pedigree	Institution developed
102	CO 8	1963	Pure-line selection from natural cross of MS6502	TNAU, Coimbatore
103	Cauvery	1962	Ullubele × H 22	UAS, Bengaluru
104	Annapurna	1962	K 1 × Aruna	UAS, Bengaluru
105	AKP 2	1962	Pure-line selection from Anakapalli local	RARS, Anakapalli
106	Udaya	1959	K 1 × Aruna	UAS, Bengaluru
107	Poorna	1959	Co 1 × Aruna	UAS, Bengaluru
108	VZM 1	1958	Pure-line selection	ARS, Vizianagaram, AP
109	VZM 2	1958	Pure-line selection	ARS, Vizianagaram, AP
110	Aruna	1956	Pure-line selection from local Giddaragi	UAS, Bengaluru
111	Co 5	1953	Pure-line selection	TNAU, Coimbatore
112	Co 7	1953	Pure-line selection from Cuddapah rajanpet ragi	TNAU, Coimbatore
113	Co 1	1942	Selection from Gidda Aryam (EC 593)	TNAU, Coimbatore
114	Co 2	1942	Pure-line selection from Udumalpet ragi (EC 3517)	TNAU, Coimbatore
115	Co 3	1942	Mutant of Co 1	TNAU, Coimbatore
116	Co 4	1942	Pure-line selection from Palladam ragi	TNAU, Coimbatore
117	Hagari 1 (Farm Ragi)	1941	Mutant from Gidda Aryam	Karnataka State Department of Agriculture (KSDA), Karnataka
118	ES 11 (Gidda Ragi)	1939	Selection from Giddaragi	KSDA, Karnataka
119	ES 13	1939	Selection from Kari giddaragi	KSDA, Karnataka
120	K1 (Kolar Gidda Ragi)	1939	Selection from Kolargiddaragi	KSDA, Karnataka
121	R 0870	1939	Pure-line selection from EC 47 of Coimbatore	KSDA, Karnataka
122	CO 6	1935	EC 1540 × EC 2945	TNAU, Coimbatore
123	H 22	1918	Pure-line selection from local ragi	KSDA, Karnataka

Source: Compendium of varieties in small millets, 2014. Compiled by MVC Gowda, YA Nanja Reddy, N Pushpalatha, M Deepika, CK Pramila, and Sachin S Jadhav, Project Coordinating Unit, All India Coordinated Small Millets Improvement Project, GKVK, Bangalore

14.10.3 Breeding for Resistance to Blast Disease

Finger millet production is constrained by: several fungal diseases such as blast (*Magnaporthe grisea*), seedling and leaf blight (*Helminthosporium nodulosum*), Cercospora leaf spot (*Cercospora eleusinis*), foot rot (*Sclerotium rolfsii*), smut (*Melanopsichium eleusis*), downy mildew (*Sclerospora macrospora*), dmaping off (*Pythium aphanidermatum*), banded blight (*Rhizoctonia solani*), sheath blight (*Marasmius candidus*), leaf spot (*Curvularia lunata*), Ozonium wilt (*Ozonium taxanum*), and rust (*Uromyces eragrostidis*); bacterial diseases such as bacterial blight (*Xanthomonas coracanae*), bacterial leaf spot (*Xanthomonas eleusinae*), and bacterial leaf stripe (*Pseudomonas eleusinae*); and viral diseases such as ragi mottle streak, ragi severe mosaic, and ragi streak. However, both in India and Africa, blast disease is the most devastating biotic production constraint in finger millet. Hence, most breeding programs aim at enhancing levels of resistance to blast disease along with grain yield potential.

Dependable knowledge on physical, biochemical, and genetic basis and availability of sources of resistance to blast help enhance the effectiveness of breeding finger millet for blast disease resistance. Several researchers have unraveled mechanism of resistance to blast disease in finger millet. Thicker leaf epidermis-cum-cuticle, higher stomatal frequency and size (Sanath Kumar et al. 2002), higher peroxidase activity, higher polyphenol oxidase, phenyl alanine ammoniase, and total phenol contents (Somappa 1999), and cytoplasm granulation (Madhukeshwara 1990) were found associated with resistance to blast disease in finger millet. Both additive and nonadditive gene effects played significant role in the expression of resistance to blast disease (Seetharam and Ravikumar 1993; Ravikumar and Seetharam 1994; Byregowda et al. 1997, 1999).

14.10.3.1 Sources of Resistance to Blast Disease

Thomas (1941) was the pioneer to report the existence of variation in responses to blast disease in finger millet. Subsequently, several researchers have attempted screening finger millet germplasm accessions/landraces/varieties/advanced breeding lines and identified numerous sources of resistance to blast disease (Table 14.8).

14.10.3.2 Breeding for Resistance to Blast Disease

African genotypes such as IE 927, IE 929, IE 922, and IE 978 had high productivity potential besides resistance to blast disease. Further, Mallanna et al. (1978) found that PR 202 and IE 927 had combined resistance to both blast and sclerotium wilt diseases. Concerted efforts led to the development and release of high yielding and blast disease resistant varieties such as GPU 28 at UAS, Bengaluru, India. It is highly popular and the ruling variety in southern parts of Karnataka state. Latha

Table 14.8 Source of resistance to blast disease in finger millet

Sl. no.	Germplasm accessions/varieties	Reference
1	TAH-91-1, TAH-8	Pall (1992)
2	APV-27	Rath and Mishra (1975)
3	TE-882, IE 1941, U.47, U-10, U.45, GE.304, GE.713	Seetharam and Viswanath (1983)
4	IE.1012, HPB 96–11, MR.1, MR2, MR3	Viswanath and Lucy Chennamma (1987)
5	GE Nos. 75, 669, 866, 1309, 1407, 1409	Ravikumar and Seetharam (1990)
6	TNAU 551	Ramaswamy (1995)
7	GE.2400, 4913, 4914, 4915	Mantur et al. (2002)
8	GE. Nos. 250, 261, 263, 320, 338, 344, 352, 416, 357, 371, 383, 396, 398, 400, 406, 409	Sanath Kumar et al. (2002)
9	MR.1, GPU-56, GPU.58, VL.321	Ramappa et al. (2002a, b, c)

et al. (2005) have established reproducible protocols for in-vitro plant regeneration and genetic transformation for development of leaf blast disease resistant finger millet using particle-in low gene-mediated method.

14.11 Genomics-Assisted Breeding

Conventional phenotype-based breeding of finger millet has been effective in developing farmer-preferred traits. However, further genetic improvement to cater to the ever-changing needs of the farmers, consumers, and processing industries and to address the challenges posed by climate change requires the use of genomic tools. The genomic tools such as markers, genetic engineering, and genome editing have proved effective to enhance genetic gains per breeding cycle and unit time. Use of DNA markers in finger millet breeding research is still in infancy as they are being developed only recently. Nevertheless, sequence independent marker systems such as random amplification of polymorphic DNA (RAPD; Fakrudin et al. 2004; Das et al. 2009; Das and Misra 2010) and amplified fragment length polymorphism (AFLP; Dida et al. 2007) have been used to detect and characterize genetic variation among germplasm accessions and breeding lines. However, the information obtained from these markers is not reliable due to their poor reproducibility. Hence, sequence-dependent simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) are highly preferred by researchers owing to their simple codominant inheritance and amenability for automation and high reproducibility. Using 1740 expressed sequence tags (EST), Arya et al. (2009) developed EST-SSR markers. Recently, Hittalmani et al. (2017) based on draft whole-genome sequence have developed a large number of genomic and EST-SSR-based markers. SSR markers being easily assayable even on simple agarose system are markers of choice by the

breeders. These markers could be used in various applications in finger millet genetics and breeding research, such as in: (1) developing fingerprint to identify duplicate germplasm accessions, (2) characterizing and assessing genetic variability in working germplasm and/or breeding lines, (3) selecting genetically diverse genotype for effecting crosses to generate variability to identify genotypes with best combination of traits, (4) identifying genomic regions/quantitative traits loci (QTL) controlling economically important traits, and (5) developing fingerprint varieties for protecting intellectual property rights associated with cultivars. Identification and validation of QTL paves way for implementation of marker-assisted selection (MAS). The use of MAS is yet to be initiated in finger millet. As a prelude to implement MAS, Dida et al. (2007) generated first-ever genetic map of the tetraploid finger millet in inter-subspecies population derived from a cross between *E. coracana* spp. *coracana* cv. Okhole-1 and its wild progenitor *E. coracana* ssp. *africana* accession MD 20 using restriction fragment length polymorphism and AFLP, EST-SSR, and genomic SSR-based markers. Assignment of linkage groups to A and B genomes was performed by comparing the hybridization pattern of probes in Okhole-1, MD 20, and *Eleusine indica* acc. MD 36. The map spanned 721 cM on the A genome and 787 cM on the B genome. Such studies need to be carried out to identify and validate QTL controlling economically important traits for implementation of MAS in finger millet.

Finger millet has ten-fold higher calcium in grains compared to other cereals and relatively high levels of drought tolerance. Identification of functional validation of candidate genes/regulatory genes controlling economically important traits such as moisture stress tolerance (Parvathi et al. 2013; Parvathi and Nataraja 2017; Ramegowda et al. 2017), salinity tolerance (Ramegowda et al. 2012), calcium transport capacity (Kanwal et al. 2014), and phosphate transport capacity (Pudake et al. 2017), coupled with efficient protocol for genetic transformation (Latha et al. 2005), is expected enhance the use of precision breeding tools such as genome editing in finger millet for rapid genetic gains per selection cycle and per unit time.

14.12 Future Prospects

The expected increased incidence of existing, and emergence of new, biotic and abiotic stresses driven by imminent climate change (IPCC 2007) warrants accelerated breeding for these production constraints. There is a need for deployment of genomic tools such as DNA markers, especially SSR and SNP markers, to enhance the pace and precision of breeding finger millet. The SSR and SNP markers should be routinely used for discovery of QTL controlling economically important traits followed by genomic selection to complement phenotype-based selection to accelerate genetic gains per breeding cycle and unit time. Genome editing tools are expected to enhance genetic gains for traits controlled by functionally well-characterized genes. While we do not claim an exhaustive review, we hope that this chapter would benefit all those who are interested in finger millet breeding.

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Chapter 15
Breeding Advancements in Barnyard Millet

Salej Sood, Dinesh C. Joshi, and A. Pattanayak

15.1 Introduction

Barnyard millet (*Echinochloa* spp.) is one of the oldest domesticated millets in the semi-arid tropics of Asia and Africa. Two main species, *Echinochloa esculenta* (A. Braun) H. Scholz; syn. *E. utilis* Ohwi et Yabuno (Japanese Barnyard millet) and *Echinochloa frumentacea* Link; syn. *E. colona* var. *frumentacea* (Link) Ridl. (Indian Barnyard millet), are cultivated and grown as cereals. It is grown in India, Japan, and China as a substitute for rice under natural precipitation. It has a wide adaptation capacity and is cultivated up to a height of 2000 m above mean sea level during summer season. It has the fastest growing character among all millets and is generally cultivated in hill slopes and undulating fields of hilly, tribal, or backward areas, where crop options are limited. Both cultivated species and their progenitors are hexaploid with $2n = 6x = 54$ where $x = 9$ (Yabuno 1962, Yabuno 1966). In addition to the two domesticated species, the genus includes about 20–30 annual and perennial wild species distributed worldwide (Clayton and Renvoize 2006; Hilu 1994), many of which are able to grow in wet or well-watered situations and compete successfully with rice. Wild barnyard millet (*Echinochloa colona*) that is commonly found in rice fields used to be harvested for food during drought years in many states of India (Padulosi et al. 2009).

In India, barnyard millet is the second important small millet after finger millet having production and productivity of 87,000 tons and 857 kg/ha, respectively (Padulosi et al. 2009). In India, it is mainly cultivated in two different agroecologies, one in mid-hills of the Himalayan region of Uttarakhand in the North and another in the Deccan plateau region of Tamil Nadu in the South.

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The plant attracted some attention as a fodder crop in the United States and Japan. The crop straw is considered superior fodder to rice, oat, or timothy straw in protein and calcium content (Obara 1936). It is also a major food for waterfowl and other birds in the United States (Mitchell 1989). In recent years, barnyard millet has received attention, mainly because of its high nutritive value. Barnyard millet contains 65% carbohydrate, 11% protein, 3.9% fat, and 13.6% crude fiber. It is also an excellent source of minerals such as iron (Fe) and zinc (Zn) and antioxidative compounds (Veena et al. 2005; Watanabe 1999). Research evidences support that the low-GI carbohydrate diets help in the prevention of obesity, diabetes, and cardiovascular disease (Brand-Miller et al. 2009), but, like any other minor millets, barnyard millet grains' utilization is limited.

15.2 Domestication and Phylogeny

There was a lack of clarity in the taxonomy of genus *Echinochloa* because of inadequate understanding of the biosystematic relationship among species and intraspecific taxa. The details of the two cultivated species of *Echinochloa* are as under:

15.2.1 Echinochloa frumentacea *Link*

Echinochloa frumentacea originated in India, and possibly also in Africa. It is annually cultivated in India, Central African Republic, Tanzania, and Malawi (Doggett 1989). It is domesticated from wild species *E. colona* (L.) Link, popularly known as jungle rice. It is grown for grain, fodder, and beer, although not as extensively as in the past. Initially, it used to be confused with *E. esculenta*, from which it differs in its whitish caryopses and proportionately smaller embryos.

15.2.2 Echinochloa esculenta *(a. Braun) H. Scholtz*

Its growth habit is annual and cultivated mostly in the temperate regions (de Wet et al. 1983) of Japan, Korea, China, Russia, and Germany, and was domesticated directly from barnyard grass (*Echinochloa crus-galli* (L.) Beauv.) some 4000 years ago in Japan (Doggett 1989). Archaeological evidence suggests that it was grown in Japan as early as Yayoi period, dating back some 4–5 millennia (Watanabe 1970). It is cultivated for fodder, grain, or birdseed. It has sometimes been included in *E. frumentacea*, from which it differs in its brownish caryopses and longer pedicels.

Interspecific hybrids between *E. crus-galli* × *E. esculenta* and *E. colona* × *E. frumentacea* were found to have normal meiotic division with 27 bivalents. The crosses

between these two groups, that is, between two cultivated species (*E. esculenta* × *E. frumentacea*) are sterile and result in meiotic irregularities, univalents, laggards, and micronuclei (Sood et al. 2014). These cytogenetic evidences suggest that the hexaploid wild species *E. colona* and *E. crus-galli* are possible progenitors of *E. frumentacea* and *Echinochloa utilis*, respectively, and the two cultivated species have different genomic composition (Yabuno 1966).

Comparison of the complete chloroplast (cp) genomes of *E. crus-galli* and *E. colona* revealed that both chloroplast genomes include a total of 136 genes. However, phylogenetic analysis revealed that *E. colona* diverged between 2.65 and 3.18 million years ago from *Echinochloa oryzicola* and *E. crus-galli* (Lee et al. 2017).

There are three recognized polyploid groups of *Echinochloa* in Temperate East Asia. The details are given below.

Group	Ploidy	Species
E. oryzicola complex	Allotetraploid	*E. oryzicola* (two forms), *Echinochloa persistentia* (non-shattering form of *oryzicola*), and cultivated forms of *E. oryzicola* and *Echinochloa phyllopogon*
E. crus-galli complex	Allohexaploid	*E. crus-galli* var. *crus-galli*, *E. crus-galli* var. *praticola*, *E. crus-galli* var. *formosensis*, *E. oryzoides*, *E. esculenta* (Japanese barnyard millet), and Lijiang millet (a cultivated form from China)
Echinochloa colonum complex	Allohexaploid	*E. colonum* and *E. frumentacea* (Indian barnyard millet)

15.3 Germplasm and its Characterization

Ex situ conservation is the widely used method to conserve millet genetic resources. Globally, more than 8000 accessions of barnyard millet have been assembled and conserved. The major gene banks conserving barnyard millet are presented in Table 15.1. The global collection is conserved in International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) Gene bank at Patancheru, India. Around 749 accessions of barnyard millet are conserved under medium- and long-term storage conditions.

Not only are collections, storage, and revival important, but also characterization of germplasm is much more demanding for its utilization by crop breeders; for example, accessions belonging to *laxa* race endemic to Sikkim state of India are not represented in the ex situ collections and thus need to be collected before it becomes extinct (Dwivedi et al. 2012). Phenotypic characterization of the collected materials has indicated that barnyard millet germplasm is highly diverse (Halaswamy et al. 2001; Gupta et al. 2009; Nirmalakumari and Vetriventhan 2009; Upadhyaya et al. 2016). For optimum and precise utilization of diversity for agronomic and nutritional

Table 15.1 Germplasm conserved across the globe

Country	Institute	Germplasm accessions		
		Cultivated	Wild	Total
Japan	Department of genetic resources I, National Institute of Agrobiological sciences	3603	68	3671
India	National bureau of plant genetic resources	1668	9	1677
	All India coordinated small millets improvement project, UAS, Bengaluru	988	–	988
	International crops research Institute for the Semi-Arid Tropics	749	–	749
China	Institute of crop science, Chinese Academy of Agricultural Sciences	717	–	717
Kenya	National gene bank of Kenya, crop plant genetic resources Centre—Muguga	192	16	208
USA	National Centre for genetic resources conservation (Fort Collins, Colorado)			306
Australia	Australian plant genetic resource information service, Biloela			67

Table 15.2 Phenotypic characterization of barnyard germplasm

Traits	Range		Mean	
	Hills[a]	Plains[b]	Hills[b]	Plains[b]
Days to flowering	30.8–67.9	33.2–73.2	51.2	49.6
Plant height (cm)	79.7–156.9	57.4–196.5	125.1	95.8
Basal tillers number	1.2–9.3	4.2–10.9	2.3	7.0
Culm thickness (mm)	2.5–9.2	4.9–7.2	5.4	5.5
Flag leaf blade length (mm)	12.3–31.3	127.8–287.9	22.6	207.6
Flag leaf blade width (mm)	1.4–3.0	11.3–32.0	2.1	19.9
Flag leaf sheath length (mm)	6.7–14.4	66.9–156.5	9.3	88.9
Peduncle length (cm)	8.1–29.8	7.5–27.7	15.2	14.4
Panicle exertion (mm)	−0.47–19.0	33.9–77.7	5.9	55.9
Inflorescence length (mm)	121.2–240.2	102.7–240.8	162.7	159.2
Raceme number	8.7–49.7	22.5–29.8	31.3	26.7
Number of nodes	4.2–9.2	9.4–11.3	6.8	10.2
Lowest raceme length (mm)	17.8–61.6	25.6–38.5	31.9	30.1
Five-ear grain weight (g)	4.2–24.3	–	12.8	–

[a]Sood et al. (2015b)
[b]Upadhyaya et al. (2016)

improvement, two core collections representing 56 and 89 accessions have been independently developed by Gowda et al. (2009) and Upadhyaya et al. (2014), respectively. The phenotypic variability in barnyard millet is presented in Table 15.2 (Fig. 15.1).

In a study of phenotypic characterization of barnyard millet core collection in Indian hills, Sood et al. (2015b) classified the germplasm into three groups based on

Fig. 15.1 Panicle diversity in barnyard millet germplasm

agro-morphological traits. All the Indian accessions along with accessions of unknown origin formed one group and possibly belong to the *E. frumentacea* group. The Japanese accessions formed a second group, indicating them to be of the *E. esculenta* group. The third group contained a mix of accessions from Russia, Japan, Cameroon, and Egypt. The reason for the clear separation of Indian and Japanese accessions was their trait morphology and growth habitat. The accessions of Indian and unknown origin in the first group were characterized by thicker culms, taller plants, greater number of nodes, longer flag leaves, longer inflorescence, greater number of racemes, and late maturity. The Japanese accessions in the second group, in contrast, typically had longer flag leaf sheath, longer peduncles, high panicle exertion, and early maturity. The third group comprised accessions with lower values for all traits except basal tiller number. The results of this study agreed with Wallace et al. (2015) who also obtained three groups using multidimensional scaling and principal component analysis (PCA) in single nucleotide polymorphism (SNP) data generated by genotyping by sequencing in the same core germplasm. Wallace et al. (2015) speculated that the accessions in the third group could be a result of seed contamination, but Sood et al. (2015b) proposed that the third group consists of accessions belonging to wild, weedy species of *Echinochloa.*

Precise characterization and evaluation of conserved genetic resources for traits of economic importance are very limited in barnyard millet. Large holdings in gene

banks and nonavailability of precise evaluation data on traits of economic importance limit the use of germplasm (Upadhyaya et al. 2009). The major impact of barnyard millet germplasm use in India is identification of Japanese barnyard millet cultivar PRJ 1 from Uttarakhand hills. In general, Japanese barnyard millet is high yielder than Indian barnyard millet in the hills, which have been observed with the release of PRJ 1 variety in the state of Uttarakhand. PRJ 1 yielded 40% higher in comparison to the Indian barnyard millet control genotypes. However, Japanese barnyard millet crop totally fails when sown late in the hills, while Indian barnyard millet gives some yield even under late sown conditions.

Halaswamy et al. (2001) characterized the national collection and identified promising entries for higher plant height (seven accessions), higher number of basal tillers (nine accessions), longer inflorescence (ten accessions), and early flowering (27 accessions). State-wise grouping of morphological characters of Indian collections of seven states did not reveal concentration of any group to a specific state (Halaswamy et al. 2001). In a similar study, the genotypes of different places clustered together cutting across distant places suggested that geographical isolation was not the only factor causing genetic diversity (Mehta et al. 2005, 2007). Similar results were also observed by Prabha et al. (2010) where the accessions of two different species clustered together, although they also found two different clusters of the accessions of two species with the help of isozyme markers. Principal component analysis of quantitative data classified the three clusters obtained from plotting first two PCA components into races *robusta*, *intermedia*, and *stolonifera* because of resemblance of morphological characters with these races; however, race *laxa* was absent in the group (Gupta et al. 2009). Association analysis results of various studies indicate that more attention should be paid to genotypes with thick culms, more number of racemes, longer inflorescence, taller plants, longer flag leaves, along with low or optimum number of tillers per plant to obtain high yield in the crop (Sood et al. 2016).

15.4 Major Breeding Objectives

The crop is still considered as a minor food and feed crop of rural low-income communities, and has not attracted breeding efforts like major cereals. Barnyard millet is less adapted to the modern agroecosystems, which leads to a different primary set of breeding targets compared to major crops. Major breeding objectives include reduced plant height and easy dehulling type. Genotypes with plant height of about 120–130 cm could minimize the problem of lodging and give required strength to the culm (Sood et al. 2015a). Barnyard millet stover is as important as grains and at higher elevation of hills, it is an important and cheap source of fodder. For instance, in Uttarakhand (India), barnyard millet contributes 11.5% to the total fodder consumption of the state (Singh and Singh 2005). Therefore, development of dual-purpose cultivars is an important breeding target to make barnyard millet a competitive crop of modern agricultural systems. While breeding for dual type,

reduction in the fodder yield due to reduced height can be compensated by increasing number of basal tillers. Development of easy dehulling genotypes will greatly reduce the drudgery involved in postharvest processing. Easy dehulling, genotype B 29 (Gupta et al. 2015) was identified from the germplasm collection maintained at Indian Council of Agricultural Research–Vivekananda Parvatiya Krishi Anusandhan Sansthan (ICAR-VPKAS), Almora, and being utilized in hybridization program to transfer the trait into high-yielding genetic backgrounds. Besides these important traits, grain yield, grain quality, and smut resistance are important breeding targets. Higher yield can be reached by improving yield components, such as grain size, which would be additionally desirable as the very small seeds of barnyard millet are causing difficulties in sowing. Quality traits for barnyard millet include high protein, glutinous texture, puffing quality, and micronutrient density in grains. The Japanese barnyard millet (*E. esculenta*) is known to possess twofold higher grain protein compared to rice (Yabuno 1987). However, transferring high grain protein content from *E. esculenta* to Indian barnyard millet (*E. frumentacea*) is a daunting challenge due to strong crossability barrier between the two species.

15.5 Conventional Breeding Efforts

Although high degree of selfing is observed in both the *Echinochloa* species, the rate of cross-pollination is sufficient to assure gene exchange among their populations (Maun and Barnett 1986). Hybridization is tedious in barnyard millet due to difficulty in emasculation of very small florets (Gupta et al. 2006). As a result, barnyard millet breeding programs largely relied on traditional mass selection and pureline selection in the country (Table 15.3). With the optimization of hot water technique for emasculation (Sood et al. 2015a), some recent varieties (Fig. 15.2) have been developed through hybridization followed by pedigree method of breeding (Table 15.3). Least research attention due to small area of the crop is another major reason for nondevelopment of improved breeding methodologies in the crop.

15.6 Breeding for Resistance to Diseases and Insect Pests

The major biotic constraint of barnyard millet is grain smut caused by *Ustilagopanici-frumentacei*, causing crop losses in India, China, and Japan. In general, *E. frumentacea* accessions show higher incidence of grain smut while *E. esculenta* accessions are mostly resistant. Germplasm screening of *E. esculenta* has already identified resistant sources for grain smut, and two accessions (PRB 9402 and PRB 9602) were observed to be immune for the grain smut in India (Anonymous 2001). However, germplasm evaluation in two separate studies at two locations in India (Almora and Bangalore) could not find a single accession of *E. frumentacea* immune to disease (Gupta et al. 2009; Nagaraja and Mantur 2008). Therefore, opportunity

Table 15.3 Details of barnyard millet cultivars in India

Cultivar	Developed through	Yield potential (q/ha)	Year of release	Remarks
VL *Madira* 172	Hybridization of EF 2 × VHC 5205	20–22	2000	Released for the states of Uttarakhand, Karnataka, and Gujarat
Sushrutha (RAU 11)	Pure-line selection	20–22	2000	Karnataka
VL Madira 181	ECC 27 × VL 60	16–18	2001	Bihar, Karnataka, Madhya Pradesh, and Tamil Nadu
PRJ 1	Pure-line selection from IEc 542	23–25	2003	Uttarakhand
Pratap Sawan 1/ER 64	Pure-line selection	15–17	2008	Rajasthan
VL *Madira* 207	VL 172 × GECH 506	16–19	2008	All barnyard millet growing states except Tamil Nadu and Gujarat
CO 2	Pure-line selection from EF 79	21–22	2009	Tamil Nadu
DHBM 93–3	Hybridization of VL-13 × IEC-566	22–24	2016	All barnyard millet growing states
DHBM-93-2	Hybridization of EF-8 × IEC-566	25–27	2018	Karnataka
MDU-1	Pure-line selection from Aruppukkottai local	15–17	2018	Southern districts of Tamil Nadu

Fig. 15.2 Farmer field demonstration of barnyard millet variety VL 207 in Uttarakhand hills of India

exists to transfer the resistant genes from *E. esculenta* to *E. frumenacea*, but the efforts have not been successful so far (Sood et al. 2014). Apart from this, anthracnose (*Colletotrichum echinochloe*), cercospora leaf spot (*Cercospora fujimaculans*), leaf blight (*Exserohilum monoceras*), and sheath blight (*Rhizoctonia solani*) are known to cause minor crop losses in barnyard millet. Resistant sources to these diseases need to be identified in disease-screening nurseries.

Among the insect pest, shoot fly (*Atherigona falcate*) and pink stem borer (*Sesamia inferens*) are the major pests damaging the crop. Germplasm screenings have already identified resistance sources to these pests and can be used to develop cultivars with increased resistant levels (Gomashe 2015). The crop has long storage life and retains quality and is practically devoid of stored grain pests.

15.7 Modern Breeding Approaches to Accelerate the Genetic Gain in Barnyard Millet

15.7.1 Mutation Breeding

Induced mutagenesis has been effectively utilized in small millets for cultivar development and widening the genetic base. Most of the barnyard millet genotypes are non-glutinous (non-waxy), giving a poor texture to the food products. However, in Asian countries, glutinous texture like rice is preferred among the consumers. Therefore, development of waxy barnyard millet genotypes imparting glutinous texture would be highly beneficial to make it more attractive for food industry. Full waxy stable mutant lines have been developed through gamma irradiation of low-amylase landrace “Nogehie” (Hoshino et al. 2010). This effort has resulted in development of new glutinous cultivar “Chojuromochi” in Japan for popularizing barnyard millet-based food products among the consumers (Goron and Raizada 2015). Likewise, variability in agronomic traits like pant height, days to maturity, and grain yield has been generated through gamma irradiation.

15.7.2 Interspecific Hybridization: Widening the Barnyard Millet Gene Pool

Wild and weedy species of the genus *Echinochloa* harbor several economic traits that are missing in mainstream gene pool (Mandelbaum et al. 1995). For instance, inflorescence length and shape, disease resistance, and plant vigor are the traits of high economic value, but there is only little variation available in cultivated barnyard millet, particularly in *E. frumentacea* (Sood et al. 2015a). Mehta et al. (2005) emphasized interspecific hybridization program involving early maturing *E. frumentacea* and high-yielding *Echinochloa esulenta* to develop early maturing, high-yielding segregants. Efforts have been made for interspecific hybridization between

E. frumentacea and *E. esulenta*. However, interspecific hybrids between distant gene pools largely failed due to the presence of great incompatibility barriers between the species (Hilu 1994). The fertility barriers between the two species have to be analyzed and modern biotechnological tools need to be employed to overcome them.

In general, Japanese barnyard millet (*E. esculenta*) possesses more diversity for agro-morphological traits compared to Indian type (*E. frumentacea*; Sood et al. 2015b). Therefore, intraspecific hybridization between the diverse genotypes of *E. esculenta* is one potential strategy to develop transgressive segregants for agronomic traits and nutritional quality. Keeping this in view, both way crosses were attempted between the two genotypes (PRB 903 and PRJ 1) of *E. esculenta* at ICAR-VPKAS, Almora, to exploit their high-yield potential and immune response to grain smut. The effort has resulted in obtaining F_6 progenies, characterized by large panicle size (22.5–26 cm), a greater number of panicle branches (>15), medium plant height (120–148 cm), and resistance to smut compared to the parents. It has been observed that panicle of *E. esculenta* genotype (PRJ 1) was characterized by large awns. The intraspecific hybridization attempt resulted in development of stable awnless segregants in the genetic background of PRJ 1, which are more vigorous than parental line (Fig. 15.3) (Anonymous 2019). These *E. esculenta/E. esculenta* derivatives with reduced plant height with uncompromised fodder potential are the positive developments toward generating dual-purpose genetic material of barnyard millet. The successful attempt allows the consideration that hybridization between *E. esculenta* genotypes will be one of the directions of barnyard millet breeding to maximize its yield potential.

15.7.3 Genomics-Assisted Breeding for Trait Improvement

Because of its status as an orphan crop and small research community, genomic resources are poorly developed in barnyard millet. One of the essential requirements of genomics-assisted breeding (GAB) is availability of a well-assembled reference genome of the crop plant. To the best of our knowledge, there is no genome sequence information available in barnyard millet. However, full genome sequence information is available for its close relative foxtail millet (Hamilton and Buell 2012). Using full sequence information of well-explored plant genomes like foxtail millet, it is now possible to conduct comparative genome analysis to detect rare valuable alleles influencing complex quantitative traits in lesser explored crops like barnyard millet. In addition, recently developed expressed sequence tags (EST) in one of its wild relative *E. crus-galli* through extensive transcriptome profiling of herbicide tolerance might be used to study the sequence information of cultivated species, particularly in *E. esculenta* (Li et al. 2013; Yang et al. 2013).

Different genome-wide markers have been employed to explore the diversity and phylogenetic studies in barnyard millet. However, these are mainly restricted to utilization of traditional non-reproducible DNA markers (Altop and Mennan 2011;

Fig. 15.3 Promising F_6 progenies of *E. esculenta* × *E. esculenta* cross: (**a**, **b**) progenies with reduced height, larger and broader leaves, and panicle size; (**c**, **d**) large awnless panicle of *E. esculenta* in the genetic background of PRJ 1

Prabha et al. 2012; Dvorakovaa et al. 2015). A number of studies reported high degree of variability in Indian barnyard millet (Hilu 1994), which are in agreement with the high degree of morphological variability observed in the species (de Wet et al. 1983). Unlike major crops, the abundant and codominant microsatellites (Nozawa et al. 2006) and single nucleotide polymorphism (SNP; Wallace et al. 2015) are rare in barnyard millet. Ability of microsatellite markers have been demonstrated in classification of and understanding the phylogenetic relationship between three agronomically important species (*E. crus-galli*, *E. colona*, and *E. crus-pavonis*) of this complex genus (Danquah et al. 2002). One potential strategy to enrich the microsatellite markers in barnyard millet is to employ the expressed sequence tags (EST) available in its taxonomically close species like finger millet and barnyard millet. Utilizing this approach, recently 51 EST-simple sequence repeats (EST-SSRs) developed from transcriptome sequence analysis were found informative in classifying the barnyard millet genotypes of South Indian origin (Manimekalai et al. 2018). Likewise, high cross transferability (>60%) of EST-SSRs derived from closely related genera such as foxtail millet (Kumari et al. 2013; Pandey et al. 2013), sorghum (Yadav et al. 2014), rice (Yadav et al. 2014; Babu et al. 2017, 2018a), and maize (Babu et al. 2018b) to barnyard millet has been reported.

Recently, the low-cost genome sequencing technologies have facilitated development of abundant next generation SNP markers, in crops with small genomes. These markers are particularly useful in assessing allelic diversity of diversity panels and core collections and use this diversity to identify genomic regions (quantitative trait loci, QTLs) through genome-wide association studies (GWAS). Such developments are not reported in barnyard millet but these will greatly help to decipher the genetic control of complex quantitative traits. For instance, detection of SNPs in genotypes bearing contrasting differences for gluten texture (waxy locus) and micronutrient density in grains will be of great significance in nutraceutical development through GAB in barnyard millet.

Well-saturated genetic maps are another vital genomic resource for detailed analysis of association between QTL and trait of interest and its subsequent introgression in desirable genetic backgrounds. To date, no genetic maps have been reported in barnyard millet. The scarcity of genetic maps is due to the lack of robust biparental and multi-parental mapping populations, which may be attributed to cumbersome crossing and limited breeding efforts in barnyard millet. Therefore, a current need in barnyard millet genomics is development of robust genetic mapping population at an accelerated pace. With the optimization of hot water emasculation technique (Sood et al. 2015a) and isolation of genotypes (IEC 566 and IEC 566/2) producing abundant pollen grains, open for long hours and protrude enough to facilitate emasculation and pollination from germplasm collection (Nirmalakumari and Vetriventhan 2009), mapping populations might become available in barnyard millet.

15.7.4 Genetic Transformation for Gain and Loss of Gene Function

The success of utilizing genetic transformation technology in any crop largely relies on efficient in vitro regeneration techniques. Regarding in vitro culture of barnyard millet, callus and plant regeneration in *E. frumentacea* (Talwar and Rashid 1989; Sankhla et al. 1992; Bobkov 2005), *E. crus-galli* (Gupta et al. 2001), and *E. colona* (Tyagi et al. 1985; Samantaray et al. 1995, 1996, 2001; Rout et al. 1997) have been well established. Although, there is only one preliminary study on transformation in barnyard millet (Gupta et al. 2001), this is a promising start point for further improvement in transformation frequency in barnyard millet. Further research toward establishing robust *in planta* transformation protocols will pave the way for studying gain and loss of function of agriculturally important genes in barnyard millet by circumventing the lengthy tissue culture cycle and somaclonal variations. Furthermore, exhaustive efforts toward establishing efficient regeneration protocols will open up avenues for determining gene function through precise genome editing.

15.7.5 Biofortification for Genetic Enhancement of Nutraceutical Value

Recently, the demand for barnyard millet has increased due to the highly nutritious grains and presence of strong antioxidative compounds (Watanabe 1999). High nutrient content (Table 15.4) and antioxidant effects make barnyard millet to be considered as a functional food crop (Kim et al. 2011), with the potential to be included in normal and therapeutic diet formulations (Veena et al. 2005). Gluten presence in main cereal crops like wheat makes it allergic to some people but barnyard millet grains are gluten free and therefore offer good opportunity for their use as health foods also (Hoshino et al. 2010). No major antinutritional compounds have been reported to date in barnyard millet (Dwivedi et al. 2012).

Both the cultivated species have higher protein content than their wild ancestors. The protein content in barnyard millet ranged from 11.1% to 13.9% (Monteiro et al. 1987). However, opposite is true for mineral content, which suggests that wild species might consist of greater proportion of embryo/endosperm because of selection of larger seeds that increases endosperm size (Mandelbaum et al. 1995). The barnyard millet grain contains about 65% carbohydrate, majority of which is in the form of non-starchy polysaccharide and dietary fiber. This helps in lowering of blood cholesterol and slow release of glucose to the blood stream during digestion. Barnyard millet is reported to have very low glycemic index compared to other millets (proso, foxtail, kodo, and little millets) and rice (Krishna Kumari and Thayumanavan 1998). There are several other reports suggesting that barnyard millet might be an ideal food for type II diabetics (Ugare et al. 2011; Surekha 2004; Arora and Srivastava 2002).

The above described unparallel nutritive value and bioactive flavonoids provide new avenues for nutraceutical development in barnyard millet through integrated breeding and genomics approach. However, the key regulatory genes governing traits for nutraceutical development still need to be deciphered in barnyard millet. The adequate phenotyping records pertaining to in-depth characterization of grain

Table 15.4 Nutritional profile of barnyard millet

Proximate composition (g/100 g)		Vitamins (mg/100 g)		Minerals (mg/100 g)		Amino acids (mg/g protein)	
Protein	11.0	Thiamine (mg)	0.33	Ca	22.0	Isoleucine	288
Fat	3.9	Riboflavin (mg)	0.10	Fe	18.6	Leucine	725
Ash	4.5	Niacin (mg)	4.2			Lysine	106
Crude fiber	13.6					Methionine	133
Carbohydrate	55.0					Cystine	175
						Phenylalanine	362
						Tyrosine	150
						Threonine	231
						Tryptophan	63
						Valine	388

quality are lacking for barnyard millet genetic resources. Consequently, these collections largely remain unutilized in barnyard millet biofortification. Therefore, extensive multi-locational characterization of these resources is essential for mining novel genetic variation for genetic enhancement of nutraceutical traits in barnyard millet. Genetic studies to determine the inheritance pattern of nutritional quality and nutraceutical development are yet to be implemented.

Recently, genomic selection (GS) has emerged as a potential tool to enhance the selection efficiency of complex quantitative traits with low heritability and costly to phenotype through traditional marker-assisted selection (MAS) approach. GS based on superior genomic estimated breeding value (GEBV) identify the genotypes expressing high heritability and discard the low-performing genotypes under selection. With the emerging examples of successful utilization of GS in wheat (Velu et al. 2016) and barley (Nielsen et al. 2016) biofortification, it is expected that it could largely accelerate the genetic gain for nutritional traits per generation in barnyard millet.

15.8 Current Developments

Two seed defensin proteins isolated from *E. crus-galli* differing by a single amino acid substitution were active against several phytopathogenic fungi and oomycete *Phytophthora infestans* at micromolar concentrations (Odintsova et al. 2008). Eight compounds isolated from Indian barnyard millet showed high antifeeding activity against brown plant hopper (Kim et al. 2008). There has always been thought of transferring the C_4 traits into C_3 plants. C_4 plants like barnyard millet exhibit many desirable agronomic traits such as high rate of photosynthesis, fast growth, and high efficiency in water and mineral use. Zhao et al. (2000) transformed rice lines by using spike stalk technique, a modification of pollen tube pathway of transformation, and developed a stable rice strain, RB 207, containing genomic DNAs of *E. crus-galli*. The new rice strain showed significant phenotypic differences from its parental line R 207, and amplified fragment length polymorphism (AFLP) analysis confirmed DNA bands of *E. crus-galli* in RB 207 (Xing et al. 2004). In addition, the differential protein expression observed between RB 207 and R 207 by proteomics data corresponds to proteins involved in photosynthesis and metabolism. Hence, the proteomic analysis results were also in agreement to some extent with the conclusions of AFLP analysis. However, the proteomics analysis did not find any of the *E. crus-galli* proteins in leaf and embryo tissues of RB 207 (Zhao et al. 2007). Similarly, in another study the pyruvate orthophosphate dikinase gene from *E. crus-galli* was introduced into upland rice through *Agrobacterium*-mediated transformation, but no appreciable change was observed in carbon assimilation although gene expression was confirmed by enzyme activity (Wang and Li 2008).

15.9 Future Prospects

The rate of genetic advancement being made now barring major millets is slow in all small millets including barnyard millet. The realizable genetic potential is much lower compared to other dryland crops. As a result, barnyard millet along with other small millets are lagging behind and getting more and more marginalized year after year. This trend needs to be reversed and breeders should intensify efforts to improve productivity along with resilience to adjust to adverse climates, which is unique to small millets. On a worldwide scale, barnyard millet like all other small millets has lost its importance as a food crop in competition with major cereals such as wheat, rice, maize, and sorghum. The decline in production has resulted in reduced consumption, which could also be attributed to changing lifestyle and government policies. Drudgery associated with processing of the crop has also resulted in decline in area and production of the crop. However, because of its short crop cycle and the fact that it can be grown on a wide range of soil types, it may remain a useful crop in Asia on poor agricultural land in regions with low rainfall or a short growing season. The prospects for Barnyard millet in India seem limited, but it may gain importance as a niche crop in dry regions at medium to high altitudes under changing climate conditions. Collective actions including public awareness on nutritional value, enhanced research on issues associated with crop production, processing and utilization, value addition, and government support for marketing could only save the crop for future.

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Chapter 16
Sorghum Improvement Through Efficient Breeding Technologies

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16.1 Introduction

Agriculture has been challenging ever since it was practiced by man. Even in the modern days, crop breeders have to face challenges in improving crops to suit demand and different environmental conditions. The succession of strategies and deployment of technologies in crop improvement were similar in almost all the crops, depending on the importance of the crop. Sorghum is among the five important crops that provide food and nutritional security to the world's population, with the other crops being maize, rice, wheat, and barley.

Sorghum [*Sorghum bicolor* (L.) Moench] is cultivated over 45.38 million ha globally, which accounts for 6.37 million tons of grains (FAO 2018). It is both a tropical and sub-tropical crop and is cultivated in about 105 countries around the world, with Asia and Africa contributing almost 80% to the total cropped area. It is also the fifth major cereal crop in the world, mainly cultivated in arid and semi-arid regions of the world. Sorghum being a C4 crop has in-built moisture and heat stress tolerance, making it a viable solution under the current climate change scenario. In

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the present global agrarian conditions, sorghum is full of opportunities. The history of sorghum improvement dates back to time immemorial; however, due to the lack of systematic breeding efforts and unavailability of records, it is unknown how the locals and landraces evolved from the existing huge variability. Nevertheless, the records in the US suggest that the introduction of sorghum dates back to the mid-nineteenth century. Many African introductions and their derivatives have ruled the US farm lands till 1924 when the conscious breeding efforts and crossing program started. The first released variety of sorghum was in the year 1923, combining drought tolerance and improved yield (Doggett 1988). The accumulation of knowledge on yielding ability (heterosis) and combining traits in crosses have led to the development of the single cross hybrid, and the first hybrid seed was sold in the year 1956 (Smith and Frederiksen 2000).

The early phase of the sorghum breeding programs mostly relied on purification of indigenous landraces or introduction through mass selection. Conscious crossing and selection among the progenies and multi-location evaluation were followed for several years until male sterility system was discovered (Doggett 1988; Smith and Frederiksen 2000). House (1985) published methods and procedures in sorghum breeding to educated breeders and technicians in sorghum improvement which included procedure for germplasm maintenance and nomenclature of breeding lines. Murty and Govil (1967) defined 70 groups in the genus *Sorghum* known as the "Working Groups," which is a modified form of Snowden's classification. This classification was more precise to explain the variation within the chosen race. Classification and characterization of germplasm pave the foundation of breeding program.

Sorghum is of different types: grain sorghum, forage sorghum, sweet sorghum and high biomass sorghum. The cultivated sorghum (*Sorghum bicolor*. M) is classified into five basic races *bicolor*, *guinea*, *caudatum*, *kafir*, and *durra* and ten intermediate races between them (Harlan and de Wet 1972). India harbors unique ecology for grain sorghum, which is cultivated in two seasons, rainy (at the onset of monsoon that is during June–July) and winter (September–October). The germplasm base of these two kinds is entirely different from each other, with the rainy season sorghum photo period insensitive and the latter being photo-period-sensitive. India is a secondary center of diversity for sorghum. Although the crop originated in Africa in the regions, including Ethiopia, Sudan, and East-Africa (Vavilov 1951), immense diversity exists in India in the form of landraces and wild relatives. Sorghum was grown in India for food, fodder, and feed since time immemorial. There are evidences that sorghum was grown in India as early as 2000 BC (Allchin and Allchin 1982; Kajale 1990, 1991; Rowley-Conwy et al. 1997; Weber 1998; Kimber 2000). All types of sorghums, viz. *bicolor*, *durra*, *kafir*, *caudatum*, and *guinea*, are found in India. Each type was used for a specific end use. The *bicolor*-types are the photosensitive type, which are primarily grown in the rainy season at the onset of monsoon, whereas the majority *durra* types grown specifically for food are cultivated during post-rainy season. The dry stalk of post-rainy season sorghum is used as fodder, which helps in sustaining the farm animals during winter and summers in peninsular India.

The Indian Council of Agricultural Research (ICAR) initiated a crop-based coordinated research programme under which All India Coordinated Research Project (AICRP) on Sorghum started in 1962. The coordinated program focused on the release of widely adapted high yielding disease and pest resistant sorghum cultivars. Soon after the inception of AICRP on sorghum, in 1962 the first hybrid CSH 1 was released. Post-1962, the sorghum research was strengthened with more researchers and centers to work. It is important to mention that the Indian program was much aggressive in sorghum improvement than any other national agriculture research programme in the world. CSH 41 (Jaicar Gold/SPH 1820) is the recently released hybrid from ICAR-IIMR (Indian Institute of Millets Research) and is a stable, high-yielding, non-lodging, medium-maturity (105–106 days), medium-tall (188 cm), and medium-bold (2.92 g/100 grains) hybrid. It has shown an average grain yield of 47.34 q/ha in all India testing (Fig. 16.1) Adoption of hybrids was quick and the system was flexible for end-use diversification of sorghum like forage, sweet sorghum, and high biomass sorghum. At present, the sorghum improvement program is headed by ICAR-Indian Institute of Millets Research located in Hyderabad, India. It has reoriented its program to diversified end uses other than grain sorghum over the period of time, like forage, sweet sorghum, and high biomass sorghum (Vilas et al. 2011).

In the year 1972, International Crops Research Institute for the Semi-arid Tropics (ICRISAT) was established in Hyderabad, India. The role of ICRISAT in global sorghum improvement program is significant, as it holds about 39,092 accessions of sorghum germplasm collected globally, with the major portion of these collections from India and African countries. The systematic characterization of germplasm

Fig. 16.1 CSH 41, a high yielding sorghum hybrid (Source: ICAR-IIMR)

and its exchange with the partners has tremendously helped national agriculture research systems globally, to develop productive cultivars. Later, in 1979 the International Sorghum and Millet (INTSORMIL) was started in the US. These two programs have helped many African and Asian countries to develop potential cultivars suitable to local environments having resistance to biotic stress (striga, grain mold, downy mildew, charcoal rot, shoot fly, stem borer, midge, head bug, etc.) drought tolerance and a wide range of adaptability.

The succession in sorghum improvement program was in phases, with new studies made to devise novel methods of genetic improvement. At every given point from the history till date, breeders were in quest to enhance the efficiency of selection or improving genetic gains per cycle of selections. Modern tools and techniques were used to complement and to integrate the factor of precision in conventional breeding program like markers-assisted selection and quantitative trait loci (QTL) mapping, mapping of genes using association mapping, development of broad-based population and simultaneous mapping as in nested association mapping (NAM), deployment of prediction models using marker information in genomic selection (GS), transgenic breeding, and gene editing technologies. Apomixis and mutation breeding were robust and are potential breeding methods; however, they did not leap beyond the conceptual stage to make an impact in sorghum improvement.

16.2 Harnessing Natural Variability in Sorghum Improvement

Landraces were the first-hand genetic resources that were improved for higher yield and disease resistance. In general, landraces harbor novel genes that provide fitness and adaptiveness to a specific region. They evolve through continuous propagation or cultivation. Initially these landraces formed the base population for improvement, while mass selection was exercised to bring uniformity in maturity, seed color, and many other traits that were important to designate as a variety in a population. Rapid gains were observed under mass selection in the early phase of systematic breeding in sorghum. In the US, the cultivars developed in early 1900s were mostly the "farmer's selection." Thirty introduction and sudangrass formed the basis of complete sorghum genetic base of the US, and the improved breeding lines became source population for hybrid breeding program. The two introductions, Feterita and Hegari from Sudan and Pink Kafir from South Africa, were very successful in the US.

Plant breeding efforts in sorghum were still in its infancy, when there was a surge in the development of new cultivars during 1910–1916. This was not termed mass selection; however, it was attributed to the cross-pollination between different cultivar types that resulted in a prolific source population (Vinall and Edwards 1916). Selection and purification of dw_1 (Karper and Quinby 1946), a natural mutant from Milo, were widely accepted starting from 1906 to 1920s. The short-statured derivatives were not goose-necked, which helped in harvesting, and they had further sur-

vived better in stressful seasons than the Standard Milo. Finnell (1929) observed that the second height mutant dw_2 and later, the double dwarf Yellow Milo, were an important cultivar in South Texas (Doggett 1988). The introduction of African Kafir in the US dates back to 1890s and later in the year 1924 the derived varieties Blackhull Kafir and Standard Blackhull covered nearly 700,000 ha in Oklahoma and Kansas states. The sorgo or the sweet sorghum was among the first introductions to the US, while the "Chinese Amber" was introduced from France in 1851. The 20 sorgos introduced in the US essentially formed the whole of sweet sorghum grown in the US. "Sart" was introduced from Sudan in 1951 and was found very promising for syrup production. The sweet sorghum was used for extracting sugar syrups. The sudangrass from Sudan was introduced as a forage type. The Sunrise Kafir was considered as a standard of forage production (Finnell 1930) and was grown widely in 12 states in the US. This non-hybrid cultivar was reported under cultivation till the end of the twentieth century. This cultivar was traced to one of the introduction lines from Natal in 1857 (Vinall et al. 1936).

The next phase of sorghum breeding by combining traits through deliberate hybridization and selection resulted in some of the earliest crosses made in 1914. The natural cross pollination between the Karif and Milo gave higher yields and was amenable for mechanical harvest. These observations encouraged sorghum breeders to undertake hybridization for combining traits for better yield and agronomy. Some of the improved varieties from the introductions formed the base genetic material for hybridization. The Dwarf Yellow Milo and the Blackhull Kafir were the two varieties released from the cross between Feterita and Blackhull Kafir. This combination has also given two other varieties, Chiltex, which was drought tolerant and Premo, a high yielder. And thereafter, several more varieties were released, viz. Bonita, Quadroon, Beaver, and Martin. The hybridization and selection efforts were taken extensively during 1920s and 1930s, which produced many potential varieties until the hybrid breeding was initiated. The expression of heterosis for yield in the hybrids was very convincing (Conner and Karper 1927; Karper and Quinby 1937); however, exploitation of heterosis in hybrids was not considered till the varieties reached yield plateau. Hot water emasculation was found not suitable for large-scale hybrid seed production, while cytoplasmic male sterility (CMS) (ms_{c1}) was found to be promising in the development of hybrid seed on a commercial scale (Stephens and Holland 1954). This marked the initiation of hybrid breeding program in the US and was soon followed in Australia in 1968. The Australian sorghum program was dependent on the US program, with most of the improved varieties introduced from the US.

Unlike that in the US, there is little information available on how the local germplasm contributed to the breeding of new cultivars in Asia and Africa. In India, two post-rainy season varieties that are very popular among the farmers for its taste and bread quality are "Maldandi" and "M35-1." The origin of Maldandi, a local landrace, is still under question; however, its derivative M35-1 (in 1937) covers 80% of total sorghum acreage in the post-rainy season (Rakshit et al. 2014). However, there is a complete and detailed account of sorghum breeding available after the inception of AICRP on Sorghum in India. The coordinated research program is strengthened

to make conscious efforts in sorghum improvement not only restricting to yield but also including agronomy, crop protection and crop physiology aspects of the crop. As a result, the sorghum improvement program in India witnessed steady growth in yield. Systematic hybrid breeding program has given several high yielding varieties and hybrids in India (Vilas et al. 2011). The research efforts in hybrid development for rainy season and varieties for post-rainy season through ICRISAT and INTSORMIL were instrumental in enhancing the average yields of sorghum in African countries. The major focus was utilizing the improved breeding lines and releasing high-yielding cultivars (Reddy et al. 2008).

16.3 Hybrid Breeding in Sorghum

Over the years, as the varietal program in the US began to witness yield plateau, the next move was to exploit the heterosis using CMS system. The landmark discovery of cytoplasmic male sterility in sorghum enabled breeders to increase yielding ability (Quinby and Schertz 1970). The CMS system opened an avenue for large-scale hybrid seed production. Unlike the varieties, the hybrids were uniform in appearance and maturity. Rapid gain was observed in the initial hybrid phase but was soon hit by a plateau. These yield plateaus were becoming more difficult after 1970s in the US.

This led to a holistic hybrid approach in the sorghum improvement programs across the world. Population improvement programs through recurrent selection were initiated, with the main aim of accumulating favorable alleles in the population, which would later become the source population for extraction of potential inbred lines. Wider adaptability was another trait which was given prominence in selection of inbred lines, and multi-location nursery trials were taken up. ICRISAT initiated recurrent selection to improve a number of traits which included stability, grain quality, resistance to economically important diseases (grain mold, charcoal rot, and leaf diseases) and pests (shoot fly, stem borers, and midge) and also moisture stress tolerance. The best genetic male sterile genes for creating recombination in sorghum were ms_3 and ms_7 (Ross 1971). The first random mating sorghum population was developed by O.J. Webster in Nebraska in 1960, initially using CMS and later changing to the use of *ms3* (Andrews et al. 1977). Doggett (1972) reported early testing in first generation of selection affected 25% gain in grain yield in four sorghum populations. A maximum yield increase of 33% was observed in PRS1. Doggett and Jowett (1963, 1964) used CMS in recurrent selection of sorghum and Gilmore (1964) suggested methods of utilizing both cytoplasmic and genetic male sterility for this purpose as it had a simple inheritance and for the fact that the expression of male sterility is not affected by the type of cytoplasm (Doggett and Majisu 1968). Webster (1965) made three cycles of recurrent selection in a bulk population using CMS, and further developed a bulk population using the Coes genetic male sterility, ms_3. Nath (1977) evaluated eight populations of sorghum; RS/R, RS/B. US/R, US/B, Tropical conv., Serere Elite, Fast Lane lR9, and Fast

Lane "B" after two cycles of selection. Six of the eight populations showed positive increases in the mean yield ranging from 21% to 36%. Only US/R and Fast Lane "R" populations gave reduced grain yields of 8% and 11%, respectively. The plant height of all the populations was reduced and grain color improved from brown to white. There was almost no change in maturity. Obilana and El-Rouby (1980) used recurrent mass selection for improving yield of two random-mating populations of sorghum, B and Y composites, and obtained yield gains of 12.8% and 13.5% cycle, respectively. Parallel to population improvement A/B line conversion program, diversification of restorers was also taken up. Some of the popular Indian R lines with good combining ability for yield are AKR150, RS585, C43, CB11, CB33, RS29, Indore 2, AKR354, etc.

Another way to break the yield plateau is by breeding for resistance to biotic and abiotic stress tolerance using emerging molecular biological techniques. However, sorghum researchers relied on techniques to study gene action for biotic and abiotic stress tolerance. The most common mating designs used were diallel and line X tester to study combining ability. Most of the quantitatively inherited traits showed predominance of additive gene action. The studies resulted in identification of good general combiners. A good combiner for shoot fly resistance was identified and a negative general combining ability (GCA) effect was reported to be desirable for a number of plants with shoot fly eggs, the number of shoot fly eggs/plant, shoot fly dead-hearts, leaf glossy score, plant vigor score, and leaf sheath pigmentation and a significant positive GCA effect for trichome density (Sharma et al. 1977; Aruna and Padmaja 2009).

Forage sorghum lines with good combining ability for different agronomic characters were developed by Paroda and Lodhi (1981). Singh and Shrotria (2008) reported non-additive gene action for leaf area, total soluble solids, crude protein (CP) content, dry matter digestibility, and hydrocyanic acid (HCN). Gene action governing forage traits like forage yield, its quality characters, and resistance to major insect pests and foliar diseases were extensively studied (Lodhi and Dangi 1981; Grewal et al. 1987; Het Ram and Lodhi 1992). Pandey and Shrotria (2009) reported non-additive gene action for dhurrin (HCN), which is reflected by high specific combining ability (SCA) in forage sorghum. In India, ICAR-IIMR recently released two varieties of forage sorghum. One variety is CSV 38F (Jaicar Hariyali/SPV 2316), which is a single cut forage sorghum. It possessed a mean of 465 q of green forage yield per hectare and possessed 7.4% protein (dry weight basis) and higher digestibility of 56.7% (Fig. 16.2) and another variety is CSV 33MF, which is a multi-cut forage sorghum with great potential to significantly enhance fodder production in the irrigated cultivation. It can produce 1039.31 q/ha green fodder from three cuts and is moderately resistant to pests and foliar diseases. It is characterized by profuse tillering and excellent regeneration after each cutting (Fig. 16.3).

The promising lines were test crossed with a series of potential restorers to produce hybrids. These hybrids were extensively tested in multi-location trials for identification of stable and high yielding hybrids. In India, entries from public and private sectors are tested in AICRP multi-location trials. Separate trials are conducted for grain, forage, high biomass and sweet sorghum. The promising entries

Fig. 16.2 CSV 38F, a single cut forage sorghum variety

Fig. 16.3 CSV 33MF, a multi-cut forage sorghum variety

with a minimum of 10% superiority over the best check are promoted to the subsequent trials. Three years of testing is done prior to preparation of release document. Based on the performance of an entry in the all India multilocation trials and additional advantage over the existing cultivars under cultivation, the entry is released for commercial cultivation. Each National Agricultural Research System (NARS) follows *sui-generis* system of releasing cultivars in their respective country.

16.4 Utilization of Trait Specific Genes in Sorghum Breeding

Classically, traits are classified under monogenic (or oligogenic) and polygenic. Every trait is manifested as a cascade of gene regulation responding to the external and intrinsic cues. The external cues include environmental factors like light, temperature, humidity, pathogen invasion, and pest infestation that are external to the plant system. The intrinsic cues are the ones inside the living system like growth and developmental stage These two signaling systems produce a phenotype. We call a trait monogenic if one or few genes in the pathway profoundly affect the expression of that particular trait. On the contrary, the genes governing polygenic traits have small and cumulative effects and are highly influenced by environmental conditions, showing complex inter- and intra-allelic interactions.

This section will describe some of the genes that were identified controlling or affecting a phenotype to a great extent. These genes have been utilized in breeding programs to be introgressed in the desired background through backcross breeding or through simple pedigree selection.

16.4.1 Maturity

Four major genes (Ma_1, Ma_2, Ma_3, and Ma_4) were identified controlling maturity during early phase of sorghum breeding (Quinby 1967). These show simple inheritance and have multiple alleles and inter allelic interaction (Table 16.1). The tropical sorghum has all dominant alleles. Later, Rooney and Aydin (1999) identified Ma_5 and Ma_6 dominant loci that are known to control photo-period sensitivity.

16.4.2 Plant Height

There are four major genes identified *dw1*, *dw2*, *dw3,* and *dw4* controlling plant height in sorghum. These genes in dominant condition produce tall plants and found to have partial dominance over the recessive counterpart. These genes are independent and show non-equal cumulative effect (Table 16.2).

Table 16.1 Sorghum varieties, maturity genes and allelic effect on days to flowering

Variety	Genotype	Days to flower
100-day Milo (100 M)	*Ma1 Ma2 Ma3 Ma4*	90
90-day Milo (90 M)	*Ma1 Ma2 ma3 Ma4*	82
80-day Milo (80 M)	*Ma1 ma2 Ma3 Ma4*	68
60-day Milo (60 M)	*Ma1 ma2 ma3 Ma4*	64
Sooner Milo (SM100)	*ma1 Ma2 Ma3 Ma4*	56
Sooner Milo (SM90)	*ma1 Ma2 ma3 Ma4*	56
Sooner Milo (SM80)	*ma1 ma2 Ma3 Ma4*	60
Sooner Milo (SM60)	*ma1 ma2 ma3 Ma4*	58
Ryer Milo (44 M)	*Ma1 ma2 ma3 R Ma4*	48
38-day Milo (38 M)	*ma1 ma2 ma3 R Ma4*	44
Hegari (H)	*Ma1 Ma2 Ma3 ma4*	70
Early Hegari (EH)	*Ma1 Ma2 ma3 ma4*	60
Combine Bonita	*ma1 Ma2 Ma3 Ma4*	62
Texas Blackhtill Kafir	*ma1 Ma2 Ma3 Ma4*	68
Combine Kafir-60	*ma1 Ma2 Ma3 Ma4*	59
Redlan	*ma1 Ma2 Ma3 Ma4*	70
Pink Kafir C1432	*ma1 Ma2 Ma3 Ma4*	70
Red Kafir P119492	*ma1 Ma2 Ma3 Ma4*	72
Pink Kafir P119742	*ma1 Ma2 Ma3 Ma4*	72
Kalo	*ma1 ma2 Ma3 Ma4*	62
Early kalo	*ma1 Ma2 Ma3 Ma4*	59
Combine 7078	*ma1 Ma2 ma3 Ma4*	58
TX414	*ma1 Ma2 ma3 Ma4*	60
Caprock	*ma1 Ma2 Ma3 Ma4*	70
Durra P154484	*ma1 Ma2 ma3 Ma4*	62
Fargo	*Ma1 ma2 Ma3 Ma4*	70

Source: Quinby (1967). The maturity genes of sorghum. Adv. Agron. 19: 267–305

Table 16.2 Height and days to bloom in some sorghum varieties (Chillicothe, Texas)

Entry	Cultivar	Genotype	Days to bloom	Height (cm)
Recessive for one gene				
Pl 54,484	Durra	Dw1 Dw2 Dw3 dw4	62	159
PI 35038	Sumac	Dw1 Dw2 Dw3 dw4	75	166
SA 1170	Tall white sooner Milo	Dw1 Dw2 Dw3 dw4	62	127
Cl 556Durra	Standard broomcorn	Dw1 Dw2 dw3 Dw4	74	207
Recessive for two genes				
SA 5155-31-29	Dwarf white sooner Milo	dw1 Dw2 Dw3 dw4	61	94
FC 8962	Texas Blackhull Kafir	Dw1 Dw2 dw3 dw4	74	100
SA 367	Dwarf yellow Milo	dw1 Dw2 Dw3 dw4	83	106
Recessive for three genes				
SA 7005	Plainsman	dw1 Dw2 dw3 dw4	64	52
SA 292	Double dwarf yellow Milo	dw1 dw2 Dw3 dw4	83	60

Source: Karper R, Quinby J (1954) Inheritance of height in sorghum. Agron. J. 46: 211–216

16.4.3 Male Sterility

Both genetic male (GMS) sterility and cytoplasmic male sterility (CMS) are reported to be operating in sorghum (Tables 16.3 and 16.4). In the sorghum population improvement program ms3 and ms7, genetic male sterility system was used to cause random pollination in recurrent selection program (Doggett 1968), whereas, the CMS system was intensively applied in sorghum hybrid seed production.

16.4.4 Brown Mid Rib

The brown midrib (bmr) mutants in sorghum were first described by Porter et al. (1978) that it causes lower accumulation of lignin. It is an important trait in forage sorghum as it increases digestibility, and bmr mutants accumulates less lignin in the plant (Saballos et al. 2008). The bmr mutants also contain lower levels of ferulic acid bridges, leading to elevated digestibilities of their stems (Lam et al. 1996). However, it is reported that to be negatively associated with forage yield and lodging (Oliver et al. 2005). There are two mutants *bmr6* and *bmr12*, which are extensively used in forage breeding. Apart from them, a few more are reported to exist (Xin et al. 2008, 2009). Recently ICAR-IIMR released a variety of Jaicar Nutrigraze (CSV43BMR/SPV2018) with higher digestibility of stover due to low lignin content. This variety is characterized by brown midrib traits due to lower lignin content, lower methane emissions for mitigating the Green House Gases (GHG) emissions, thus protecting the environment (Fig. 16.4).

Table 16.3 Genetic male sterility genes and their designated symbols and mechanism of sterility

Gene symbol	Mechanism	References
ms_1	Normal pollen is dominant over aborted or empty pollen cells	Ayyangar and Ponnaiya (1937a, b)
ms_2	Normal pollen is dominant over aborted or empty pollen cells	Stephens (1937)
ms_3	Normal pollen is dominant over aborted or empty pollen cells	Webster (1965)
ms_4	Empty pollen cells	Ayyangar (1942)
ms_5	Aborted pollen	Barrabbas (1962)
ms_6	Micro anthers without pollen	Barrabbas (1962)
ms_7	Empty pollen cells	Andrews and Webster (1971)
al	Antherless stamens	Karper and Stephens (1936)

Source: Rooney W (2000) Genetics and Cytogenetics: Sorghum origin, history, technology, and production, Vol 1

Table 16.4 Information on cytoplasmic male sterility (CMS) genes in sorghum

Cytoplasm fertility group	Identity	Race	Origin
A1	Milo	D	–
	IS 6771C	G-C	India
	IS 2266C	D	Sudan
	IS 6705C	G	Burkina Faso
	IS 7502C	G	Nigeria
	IS 3579C	C	Sudan
	IS 8232C	(K-C)-C	India
	IS 1116C	G	Indian
	IS 7007C	G	Sudan
	IS 1262C	G	Nigeria
	IS 2573C	C	Sudan
	IS 2816C	C	Zimbabwe
A3	IS 1112C	D-(D-B)	India
	IS 12565C	C	Sudan
	IS 6882C	K-C	USA
A4	IS 7920C	G	Nigeria
9E	IS 7218		Nigeria
	IS 112603C	G	Nigeria
A5	IS 7506C	B	Nigeria
A6	IS 1056C	D	India
	IS 2801C	D	Zimbabwe
	IS 3063C	D	Ethiopia

Source: Schertz K (1994) Male-sterility in sorghum: its characteristics and importance. In: Use of Molecular Markers in Sorghum and Pearl Millet Breeding for Developing Countries: Proceedings of an ODA Plant Sciences Research Programme Conference on Genetic Improvement, pp. 35–37
B bicolor, *C* Caudatum, *D* Durra, *G* Guinea, *K* Kafir

16.5 Modern Breeding Strategies

16.5.1 QTL Mapping Studies and Genomics

The DNA markers came handy to generate complete linkage map of sorghum. As the marker technology advanced, marker density greatly increased. The high-density maps were used for identification of QTLs and fine mapping. Better computational algorithms enabled geneticists to estimate and dissect component of genetic effects in QTL analysis (composite and interval mapping). Two decades since the QTL mapping was conceptualized, 150 QTL mapping studies were conducted identifying 6000 QTLs for 220 traits in sorghum (Mace et al. 2019).

The results from QTL mapping failed to make impact in crop improvement due to the fact that the information generated was limited to the mapping population that could not be extended to other populations, and a number of reasons are attributed that are theoretical or methodical. However, some QTLs were consistently reported

Fig. 16.4 Jaicar Nutrigraze, a special variety with higher digestibility of stover due to low lignin content

in a number of studies especially for agronomic traits, viz. yield, plant height, and maturity (Mace and Jordan 2010). But, still heterogeneity existed among QTL studies; meta-analysis methods (Goffinet and Gerber 2000; Arcade et al. 2004; Veyrieras et al. 2007) were devised to integrate genetic linkage maps and QTLs into a single reference map, and partly the method was successful in integration but the information generated through Genome-wide association study (GWAS) was not included, missing out a major proportion of QTLs and trait-associated markers. Mace et al. (2019) developed QTL Atlas integrating all the QTL and GWAS studies and projecting them on the sorghum genome sequence. It is developed with an objective to provide information on QTLs and significant marker–trait association to accelerate sorghum breeding through molecular tools. This method is expected to estimate the pleiotropic effects of QTLs that can explain physiological and functional relation/interaction. Mace et al. (2019) provide two examples: the SBI-02 had B2 for a high-tannin testa layer in the sorghum grain which is associated with grain quality QTL (Rami et al. 1998), grain mold resistance (Upadhyaya et al. 2013), grain composition (Shakoor et al. 2014; Rhodes et al. 2017) and cold tolerance (Knoll et al. 2008). The second example, chromosomal segment SBI-07, harbors dw_3 gene for plant height; the locus is also mapped for stem juice yield or shows pleiotropic effect (Guan et al. 2011; Murray et al. 2008a), panicle exertion (Zhao et al. 2016), biomass

(Guan et al. 2011), panicle architecture (Pereira et al. 1995; Brown et al. 2006) and lodging (Murray et al. 2008b). Most of the advancements in sorghum breeding and genetics leaped to new heights after sorghum genome sequence published.

Sorghum has 10 chromosomes holding approximately 800 Mbp (Price et al. 2005). The sorghum genome sequence, genotype BTx623, is the first C4 grass fully assembled using traditional Sanger sequencing (Paterson et al. 2009). There is continuous improvement in the reference genome, and new functional genes copies and accurate repetitive regions are identified and annotated (McCormick et al. 2018). To have a refined genome sequence, much deeper short read sequences (110×) was generated. Furthermore, to improve order and coverage of the reference genome, a high-density genetic map was generated using 10,000 markers 437 recombinant inbred lines (RILs) from the sorghum lines BTx623 and IS3620C. This has enabled to integrate seven sequence contigs spanning 24.64 Mbp into the v1 reference genome chromosomes which formed the version 3 of sorghum genome sequence (Truong et al. 2014). The BTx623 genome sequence represents reference genome of sorghum that has leveraged genomics, transcriptomics, and proteomics studies.

A second sorghum reference genome ("Rio") was assembled to identify the cause of sugar accumulation in sweet sorghum. Currently, there are 44 sorghum accessions that have been sequenced using whole genome sequence at a minimum coverage of 16× and are publicly available (Mace et al. 2013). As the sequencing cost reduced, a greater number of genotypes were sequenced.

16.5.2 Association Mapping

Association mapping uses a historical recombination that has formed a linkage disequilibrium block in a diverse structured population to identify the marker trait association. Association mapping could be based on candidate gene or genome wide. Candidate approach is directed in a set of candidate putative genes manifesting phenotype. With the advancement in sequencing technologies and cost-effective methods, whole genome sequencing and resequencing were possible. A new genotyping tool emerged called Genotyping-by-Sequencing (Elshire et al. 2011), which had enabled researchers to generate high-density SNP data across genome, possibly in the genic region. Then genome-wide association studies took over the candidate gene association mapping. The advantages of association mapping make them popular among the crop researchers (Bernardo 2001; Liu et al. 2003; Massman et al. 2013). In sorghum, a number of candidate genes are identified through GWAS for a number of traits, oligogenic to complex traits like yield and abiotic stress like drought and cold tolerance (Morris et al. 2013; Shakoor et al. 2014; Brenton et al. 2016; Boyles et al. 2019). The genotype-by-environmental (G × E) interactions are important when it comes to crop improvement utility of association mapping or any other cutting-edge technologies. Velazco et al. (2019) suggest the inclusion of a great number of individuals, a million for GWAS as done in humans.

Nested association mapping (NAM) combines the power of QTL mapping and association mapping, which is a remarkable resource to dissecting the genomic architecture of phenotypic variation (Yu et al. 2008). As described by Yu et al. (2008), NAM involves the following steps: "(1) selectingdiverse founders and developing a large set of relatedmapping progenies preferably recombinant inbred lines (RILs) for robust phenotypic trait collection, (2)either sequencing completely or densely genotyping the founders, (3) genotyping a smaller number of tagging markers on both the founders and the progenies to define the inheritance of chromosome segments and to project the high-density marker information from the founders to the progenies, (4) phenotyping progenies for various complex traits, and (5) conducting genome- wide association analysis relating phenotypic traits with projected high-density markers of the progenies."

Boyles et al. (2019) reviewed sorghum genetics and genomic resources for connecting genotype and phenotype where there is a mention of three NAM populations and two Back-cross NAM populations among which four focus on grain sorghum and one focuses on bioenergy sorghum. Among the five, only two of the NAMs have fully accessible seed and genomic data: the "RTx430" population (Bouchet et al. 2017) and the Australian BC-NAM population (Jordan et al. 2010).

The NAM population of 2214 RILs from ten diverse founder lines which represent global sorghum diversity and RTx430 (recurrent female parent) was genotyped and phenotyped to study the robustness of the population in dissecting complex traits. The 90,000 SNP data on the RILs revealed four to five times higher recombination rates in the coding sequence amounting to 57,411 recombinant events. The population genotyping captured 65% of flowering time genetic variance and 75% of plant height variance (Bouchet et al. 2017). Thus, concluding that NAM has power to scan full genome with high power to identify quantitative trait loci (QTL) with effects of different sizes (Yu et al. 2008).

16.5.3 Genomic Selection

Best Linear Unbiased Prediction were described much earlier than Robinson (1991). Henderson (1975) suggested the use of Best linear unbiased prediction (BLUP) and Best linear unbiased estimators (BLUEs) in selection. Later, Meuwissen et al. (2001) successfully extended to animal breeding, which is now called "Genomic Selection." Genomic selection (GS) has evolved over the Marker Assisted Selection methods with the aim to increase genetic gains per cycle of selection. The limitations in those methods were overcome to some extent. In genomic selection, markers are assigned with breeding value (genomic estimated breeding value, GEBV). The training set is a representative subset which is phenotyped and genotyped, for the estimation of true breeding value or genomic estimated breeding value (GEBV). The statistical models must be optimized for estimating GEBV for better accuracy. The optimized statistical models are employed for predicting performance in breeding set using genotyping data alone. The accuracy in prediction is based on many

factors like relationship between the training and the breeding populations, the number of generations that separate them, heritability of the trait(s), type and number of markers and, most importantly, the accuracy in phenotyping (Varshney et al. 2014).

Optimizing genomic selection study using simulation revealed that genetic gain in first few cycles for oligogenic traits was 12–88% over the phenotypic selection, whereas, in polygenic traits, maximum relative genetic gain advantage of 26–165% was observed and was always superior to phenotypic selection. The study also showed 67% reduction in cost per unit of genetic gain. In addition, updating the predictive model in every cycle has improved the genetic gains up to 39% (Muleta et al. 2019). Modifying models by including multi-trait has proved enhanced prediction ability. The multi-trait models combining grain yield, stay green, and plant height information produced the highest improvements in predictive ability for GY (up to 18%) than the single-trait G-BLUP model (Velazco et al. 2019).

16.5.4 Transgenic Approach

Sorghum is infamously known for its recalcitrance to tissue culture and regeneration. In fact, it is classified as one of the most challenging plant species for tissue culture and genetic transformation (Zhu et al. 1998). However, both particle bombardment-mediated and agrobacterium-mediated transformation methods are reported to be successful in sorghum. The first successful transgenic sorghum was developed through particle bombardment method for herbicide resistance in the year 1993 (Casas et al. 1993), followed by the first transgenic sorghum through *Agrobacterium*-mediated transformation, once again for herbicide resistance (Zhao et al. 2000). Thereafter, several modifications and improvisations in terms of methodologies, explants, genotype-specific protocols and culture conditions were reported (Elkonin and Pakhomova 2000; Nirwan and Kothari 2003; Sato et al. 2004; Gao et al. 2005a, b; Nguyen et al. 2007; Gurel et al. 2009; Grootboom et al. 2010; Liu and Godwin 2012). Of the transformation methods, *Agrobacterium*-mediated method remains the best approach while among the explants tested and tried, immature embryos are reported to be predominant over other explants like immature embryos, immature inflorescences, protoplasts, anthers, microspores, and shoot apices in terms of transformation efficiency and success rate (Belide et al. 2017). Sorghum transgenics are developed for various needs; however, improvement of nutritional value is one of the main objectives of genetic transformation of sorghum. γ-Kafirin, one of the seed storage proteins in sorghum, is highly resistant to proteases leading to the low nutritive value of sorghum and its by-products. Transgenic sorghum was developed using *Agrobacterium*-mediated genetic transformation employing RNA interference (RNAi) silencing of the γ-kafirin gene (Elkonin et al. 2016). These plants had lowered (2.9–3.2 times) and altered forms of the 28-kDa γ-kafirin protein (digestibility index reaching 85–88% as to 60% in the control). A

high-lysine protein gene HT-12 of barley was introduced into sorghum through *Agrobacterium*-mediated transformation, resulting in a 40–60% increase in the lysine content in transgenic sorghum (Zhao et al. 2002). *Agrobacterium*-mediated approach was employed to introduce the alanine aminotransferase from barley (*Hordeum vulgare*), HvAlaAT into sorghum for increased alanine aminotransferase (alt) activity. The enhanced alt activity was supposed to impact height, tillering, and vegetative biomass relative to controls. However, there were no significant phenotypic effects in sorghum, although enhanced enzymatic activity was reported in the transgenics (Pena et al. 2017). With regard to transgenic sorghum for biotic stress tolerance, particle bombardment was used for genetic transformation of sorghum genotype, KAT 412, with chitinase and chitosanase genes isolated from *Trichoderma harzianum*. Transgenic sorghum expressing the two anti-fungal genes was found to be significantly more tolerant to anthracnose than the wild type (Kosambo-Ayoo et al. 2011). Sorghum transgenics were also reported fungal resistance using chitinase gene from rice (Arulselvi et al. 2010) and American elm (Devi and Sticklen 2003). Transgenic sorghum plants expressing a synthetic *cry1Ac* gene from *Bacillus thuringiensis*(Bt) with a wound-inducible promoter from the maize protease inhibitor gene (mpiC1) was developed (Girijashankar et al. 2005) and Visarada et al. (2014) developed with two *Bt* genes (*cry1Aa* and *cry1B*) in two parental lines, CS3541 and 296B, for stem borer resistance.

16.5.5 Genome Editing

The genome editing system CRISPR/Cas9 has been recognized as a revolutionary tool for precise breeding and improvement and is widely used in plant and animal systems in the last 5 years. CRISPR, which stands for Clustered Regularly Interspaced Short Palindromic Repeats, has two molecules, the enzyme cas9 and a guide RNA. The first successful attempt at gene editing in sorghum was reported in 2013 along with rice, tobacco, and Arabidopsis (Jiang 2013) using the *Agrobacterium*-mediated transformation method. A highly efficient sorghum transformation and development of stable knockouts in sorghum using CRISPR/Cas gene editing system were reported by Che et al. (2018). This study explored a range of selectable markers for Tx430 as well as the recalcitrant Africa varieties, including Macia, Malisor 84-7, and Tegemeo for the genetic transformation and gene editing. Gene editing to reduce α-kafirin protein content was demonstrated by targeting the k1C gene family that codes for kafirin proteins. A single-guide RNA was designed to introduce mutations in a conserved region encoding the endoplasmic reticulum signal peptide of α-kafirins. T1 and T2 seeds showed reduced α-kafirin levels, and significantly increased grain protein digestibility and lysine content (Li et al. 2018). Recently, the CRISPR/Cas9 gene editing through biolistic bombardment for two target genes, cinnamyl alcohol dehydrogenase (CAD) and phytoene desaturase (PDS), in the sorghum genotype Tx430 was reported by Liu et al. (2019). Both

homozygosis and heterozygosis editing of CAD gene were confirmed in T0 primary transgenic lines through sequencing PCR products. At the same time, a comprehensive methodology applying gene editing technique using *Agrobacterium*-mediated transformation in sorghum is described by Sander (2019).

16.6 Mutation Breeding

Mutation breeding is considered as a means of corrective breeding which is used mainly for three purposes: (1) to improve specific trait(s) in an existing popular cultivar, (2) cosmetic change as a morphological marker for varietal registration, and (3) induction of male sterility or fertility restoration (Wanga et al. 2018). Mutation breeding is the easiest way of creating novel variation and has been used in many genetic studies to knock off genes. Globally, 15 sorghum cultivars were released through mutation breeding, all being radiation induced. Pahuja et al. (2013) developed SSG 59-3 mutants with improved fodder quality (low HCN, high protein, and high digestibility). Five sorghum cultivars were irradiated at 200 (gamma-rays) Gy and 300 Gy to induce mutation. The progenies were screened from M2 onwards. Variation was observed in several characters such as plant height, resistance to lodging, plant architecture, drought tolerance, panicle length and compactness, seed size and color, seed quality (vitreous or floury) and protein content, glume color and structure, flowering date (early and late maturity), and tillering capacity (Bretaudeau 1997). A significant increase in grain yield (>4.5 t/ha) was recorded under moisture stress condition in the mutants B-68, B-72, B-95 and B-100 than the original parent Durra (3.50 t/ha) and the control check varieties UPCA (2.68 t/ha) and Hegari (3.75 t/ha) (Human et al. 2012). Balakrishna et al. (2015) reported that 30 promising mutants developed in IS 18551 background through irradiation with 500 Gy gamma-rays showing resistance to shoot fly across locations (four) for two seasons. At ICRISAT, induced mutagenesis helped in reducing the height of biofortified sorghum cultivar ICSV 15013, which is currently under multi-location trial in AICRP on sorghum (Wanga et al. 2018).

16.7 Apomixis in Sorghum

In angiosperms, apomixis refers to development of seed asexually, which is regarded as exception to the natural sexual process of seed production. The unreduced cells of ovule take an alternative developmental path to produce asexual totipotent cells that could be nucellus, chalaza or integuments. These cells give embryos directly (adventitious embryony) or after $2n$ embryo sac (ES) formation (gametophytic apomixis). Embryo formation is parthenocarpic in apomictic ($2n$) whether in sexual

plants or apomicts, embryony is the result of epigenome modifications that begin as early as floral transition (Tucker and Koltunow 2009). The study by El'konin et al. (2012) revealed A3 cytoplasm-suppressed development of parthenogenetic pro-embryos but did not affect apospory-ES development. They also reported that the frequency increased with higher temperature and moisture stress. The R473 sorghum apomictic line was extensively studied; it did not set seeds when crossed with other sorghum lines (Rao and Narayana 1968). Rao and Murty (1972) and Murty and Rao (1972) observed multiple embryo-sac development and good seed set in "R 473" in spite of apparent failure of pollen to germinate on self-stigmas. They postulated that the line functioned as an obligate apomict when self-pollinated. The study by Rao et al. (1978) revealed that 90% of the pollen is fertility in R473 but only 60% germinated in culture study. The pollen tubes growth was significantly reduced and curved and blunt pollen tube tip indicated arrested growth. Using the comparative sequence, Galla et al. (2019) noticed that *Paspalum* ACR has homology to a telomeric region of chromosomes 8 of sorghum, but inverted. Murthy et al. (1972) urged the requirement of markers in this uniform line to distinguish between mature sexual and apomictic embryo sacs so that exact frequency of apomictic reproduction is established.

16.8 Way Forward

Sorghum being one of the crops of opportunities in the twenty-first century, apart from food and animal feed it is leading, is becoming an energy crop. The first- and second-generation biofuel production will be primarily dependent on sorghum in near future. Research focus in genetic improvement for higher yield and agronomic practices for clean crop is not the ultimate goal, rather it is one of the paths for better future harnessing natural resources optimally.

Most of the tools and techniques in plant breeding overviewed in the chapter are meant to complement for a better future. Identification of sources of yield, biotic and abiotic stress and quality, combining traits in breeding program and evaluation in target environment as a whole, is a continuous process. The path-breaking novelties brought out by mutation and fixation of heterosis through apomixis need focused efforts without judgment. Transgenics are a controversial subject in state affairs to which the sorghum improvement must not suffer. Gene editing is an evolving technology in sorghum, and it has opened avenues to change the phenotype which is conventionally thought to be impossible or overcoming limits of other technologies. Gene editing can be extended to manipulate homeotic and heterochronic gene, changing the active sites of rate-limiting proteins for better stability under harsh environment. Harmonizing technologies in entirety of sorghum with environment (nature) is the key for all challenges which humankind is enduring.

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Index

A
ABC transmembrane transporter, 298
Abiotic conditions, 52
Abiotic stress tolerance, barley
  drought stress, 328
  factors, 322
  freezing stress, 325–327
  heavy metal toxicity
    AI, 327
    Calvin cycle enzymes, 327
    Cd, 328
    Mn toxicity, 328
  lodging, 329, 330
  nutrient stress, 330, 331
  ROS, 322, 323
  temperature stress, 323–325
  waterlogging, 329
Accelerated breeding
  agriculture, 1
  climate change and global warming, 1
  conventional breeding approaches, 2
  embryo culture, 9
  genetic mapping, 15, 16
  genome editing, 13, 14
  GS, 12
  heat and drought stresses, 1
  high-throughput genotyping, 18, 19
  HTP, 17, 18
  innovative techniques, 2
  MABS, 15
  MAS, 14
  micropropagation, 7
  plant breeding, 1
  plant improvement, 1
  RB, 12, 13
  shuttle breeding, 11
  somaclonal variation, 8, 9
  speed breeding, 10, 11
  SSD, 16, 17
  transgenic breeding, 9, 10
Adenosine triphosphate (ATP), 320
Adult plant resistance (APR), 207
Agrobacterium-mediated approach, 427
Agrobacterium-mediated transformation, 404
Albinism, 197, 246
All India Coordinated Research Project
    (AICRP), 413
Amplified fragment length polymorphism
    (AFLP), 16, 340, 385, 404
Androgenesis, 171, 235, 242–243, 245
  albinism, 197
  DH protocols, 197
  donor plants, 195, 197
  embryogenic response, 196
  microspore culture response, 195, 196
  microspore embryogenesis, 197
  microspore isolation, 199
  ovaries addition, 199
  plantlet development, 201
  plating embryoids, 200
  spike collection, 197
  spike sterilization, 198
Androgenic DH method, 195
Androgenic methods, 234
Animal breeding technology, 53
Anther culture, 4
Anther culture/microspore culture
  androgenesis, 146
  callus/embryoids yield, 146
  comparison, 149
  induction, 147, 148
  vs. maize method, 149

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Anther culture/microspore culture (*cont.*)
pretreatment, 146, 147
regeneration, 148
Anther emergence rate (AER), 279
Anthocyanins, 320
Arabidopsis thaliana, 7
ARGOS8 protein, 303
Artificial chromosomal doubling
colchicine, 276, 278
coleoptile tip, 277
floral primordial stage, 279
herbicides, 278
N_2O gas, 278
pronamide, 278
protocols, 277
seedling immersion method, 278
Artificial electric lamps, 32
Artificial genome doubling, 249
Artificial intelligence, 98, 102
Artificial neural networks (ANNs), 107
Association mapping, 53
Automated imaging, 54
Auxin and cytokinin, 244

B

Bacillus thuringiensis (Bt), 427
Backcross breeding, 336
Barley, 33, 36
adaptability, 309
biotechnology-based/marker-assisted approaches, 337–339
biotic stresses, 316
breeding goals, 344
chromosomes, 309
cultivation, 309
domestication and cultivation (*see* Domestication and cultivation, barley)
evolution, 310
genetic bottleneck, 342, 343
genetic diversity exploration, 343
genetics, 310
H. vulgare, 310
pre-breeding, 343
production, 311, 312
species, 310
spontaneum, 310
stress tolerance (*see* Abiotic stress tolerance, barley) (*see* Biotic stress resistance, barley)
Barley Yellow Mosaic Virus (BaYMV), 315
Barnyard germplasm
accessions, 395
agronomic/nutritional improvement, 393
characterization, 393
E. esculenta group, 395
E. frumentacea group, 395
economic traits, 395
gene banks, 393, 394
genetic diversity, 395, 396
national collection, 396
PCA, 396
phenotypic characterization, 394
phenotypic variability, 394
Barnyard millet
AFLP analysis, 404
Agrobacterium-mediated transformation, 404
antioxidant effects, 403
biofortification, 404
cultivars, 397, 398
diseases resistance, 397, 399
domestication and phylogeny
E. crus-galli complex, 393
E. esculenta, 392
E. frumentacea L., 392
fodder crop, 392
genetic advancement, 405
genetic transformation, 402
germplasm (*see* Barnyard germplasm)
micromolar concentrations, 404
minerals, 392
modern breeding approaches, 399–404
non-starchy polysaccharide/dietary fiber, 403
nutrient content, 403
nutritive value, 403
production, 405
production and productivity, 391
public awareness, 405
types, 391
varieties, 397, 398
BASTA herbicide resistance, 274
Bayesian estimation, 67
Bayesian model, 70
Bayesian multi-trait and multi-environment (BMTME), 112
6-Benzylaminopurine (BAP), 147
Best linear unbiased estimation (BLUE), 70
Best linear unbiased prediction (BLUP), 67, 70
Biofortification, 293, 294
Biotechnology-based/marker-assisted approaches
agrobacterium, 338
breeding method evolution, 339
DArT markers, 337

β-glucans, 338
ICARDA, 338
molecular breeding and genomics, 341, 342
molecular markers, 337
optimal genetic complexes, 337
QTL mapping, 338
transformation efficiencies, 338
Biotic and abiotic stress-resistant crops, 66
Biotic stress resistance, barley
genetic variation, 332
pedigrees and molecular data, 331
pre-breeding approach, 331
primary germplasm pool, 331
secondary germplasm pool, 331
sources, 332–335
tertiary germplasm pool, 331
Bi-parental population, 53
Brachypodium distachyon, 36
Bread wheat (*Triticum aestivum* L.), 139
Breeding 3.0, 101
Breeding 4.0, 92, 101
Breeding activities, 301
Breeding, finger millet
Africa, 377
productivity
farmer-preferred traits, 375
GPU varieties, 376, 378
hybridization, 376
Indaf varieties, 376, 377
Madayyanagiri, 375
varieties, 376
resistance, blast
bacterial diseases, 384
genotypes, 384
sources, 384, 385
thicker leaf epidermis-cum-cuticle, 384
Breeding methods
indica rice, 225
progenies, 225
Breeding methods evolution
barley breeding via haploidy, 339, 340
genome analysis, 341
GM Barley, 341
molecular marker and MAS, 340, 341
Breeding population (BP), 53
Breeding scheme
F_2 recurrent mass GS, 63, 64
F_3 recurrent GS, 64, 65
F_4 recurrent GS, 65
F_7 recurrent GS, 65
Breeding selection, 55
Breeding techniques, barley
backcross breeding, 336
bulk handling, 332
composite crosses, 335
haploid breeding, 337
MSFRS, 335
pedigree breeding, 336
SSD, 336
Breeding tools, 218
Bulbosum method, 5, 6, 141, 170
Bulk handling, 332
Bulked segregant analysis (BSA), 129

C

Cadmium (Cd), 328
Canopy temperature, 18
Carbon isotope discrimination (CID), 324
Carbon source, 240
CAUHOI, 272
Cauvery, 376, 383
CENH3 HI system, 303
CENP-A targeting domain (CATD), 177
Centromere specific histone 3 (CENH3)
gene transcription, 176
genome elimination, 176
GFP, 177
hypothesis, 177
modification methods, 177, 178
N-terminal tail region, 176
Chromosome doubling, 145, 146, 179, 180, 193, 276
Chromosome elimination
CRISPR/Cas9 systems, 178, 179
DH, 167, 180–182
distant hybridization, 168
haploid embryos, 167
interspecific fertilization, 169
interspecific species, 169
kinetochore activity, 169
multipolar spindles, 169
targeted centromere manipulation, 176–177
uniparental/preferential, 169
wide hybridization, 170–176
CIMMYT conventional breeding methods, 202
Cinnamyl alcohol dehydrogenase (CAD), 427
Cleaved amplified polymorphic sequence (CAPS), 224
Climate change, 31, 139
Colchicine, 180, 276
Cold pre-treatment, 238
Common wheat (*Triticum aestivum* L.), 167
Community integration, 102
Composite crosses, 335
Computer vision-based phenotyping, 109

Conventional breeding programmes, 32
Conventional pedigree breeding methods, 202
conventional rice breeding, 233
Convolutional neural networks (CNN), 104
CRISPR/Cas system, 13, 302
CRISPR/Cas9-mediated genome engineering, 228
CRISPR/Cas9 systems, 178, 179
Crop improvement, 3, 11
Crop ontology (CO), 101
Crops, 33–35
C-terminal histone fold domain (HFD), 176
Culture medium, 247
Cytoplasmic male sterility (CMS), 415, 421, 422

D
DArT markers, 337
Data acquisition
  definition, 95
  environment data, 99–100
  genotype data
    cost savings, 95
    file formats, 95, 96
    global genotyping assay market, 95
    NGS, 95
    SNP, 95
    software packages and environments, 95
    speed and efficiency, 95
  phenotype
    automated phenotyping, 98
    challenges, 99
    crop phenomics, 98
    HTP phenotyping, 98
    metabolomics, 97
    micro-phenotyping, 98
    organ-level phenotyping, 98
    phenomic data, 97, 99
    proteome and metabolome, 96
    proteomic data, 97
Data analytics
  artificial intelligence, 102
  breeding pipeline phases, 102, 107
  breeding programs, 102
  genotypic, 104, 106
  phenotypic analysis, 107–111
  terminology, 102, 108–109
Data integration
  community integration, 102
  DMASTs, 101
  free and open-source tools, 102–106
  HTP genotyping, 101
  HTP precision phenotype, 101
  ontologies, 101
  traits, 101
Data management
  acquisition, 95–100
  categories, 93
  data analysis, 102–112
  data-intensive science, 94
  impacts, 94
  infrastructure, 113
  integration, 101–102
Data Management Analysis and Decision Support Tools (DMASTs), 101
Data-dependent acquisition (DDA), 97
Data-independent acquisition (DIA), 97
Data-intensive science, 94
Datura inoxia, 141
Days post anthesis/crossing (DPA), 208
Deep learning
  algorithms, 109
  annotated image dataset, 109
  digital agriculture, 112
  GS, 104
  image-based phenotyping, 109
DH line production
  haploids induction, 259–262, 286
  maize breeding programs, 286
  maternal haploid inducers, 266–269
  MHI associated traits, 262, 263
  MHI genetics, 263–265
  MHI mechanisms, 265, 266
  refinements, 286
DH-derived wheat varieties, 151–153, 155
Dimethyl sulfoxide (DMSO), 145
Distinctness, uniformity, and stability (DUS), 284
DNA-based markers, 18
DNA sequencing technologies, 61
Domestication and cultivation, barley
  abiotic stress tolerance, 316
  brittleness of rachis, 313
  disease resistance, 315
  dormancy, 314
  growth habit, 314
  history, 313
  hypothesis, 313
  kernel row type, 314
  mono-centric origin, 311
  mono-phyletic origin, 313
  naked/covered kernel, 314
  productivity and quality traits, 315
Double fertilization, 263, 266
Doubled haploid (DH), 123
  advantage, 301

anther culture, 4
anther culture/microspore culture, 146–149
Bulbosum method, 5, 6
chromosome elimination, 167, 180
CIMMYT's protocol, 301
conventional breeding, 2
cross-pollinated crops, 2
development, maize, 301
diploids, 248
gene engineering, 7
genetic mapping, 3
genotypes, 301
grain quality, 250
heritable traits, 180
in vivo, 6
inducer lines, 6
isolated microspore culture, 4
maize method (*see* Maize method)
MAS, 168
ovary culture, 5
PHB71 and KRH2, 250
plant breeding, 181, 182
plant-breeding programmes, 233
population, 181
pre-breeding programmes, 182
production, 234
public and private sector organizations, 3
QTL mapping, 181
self-pollinated crops, 2
transgenic embryos, 181
wheat breading (*see* Wheat breeding)
wheat × *Imperata cylindrica*, 6
wheat × maize system, 6, 181
whole genome elimination, 181
Double-digest restriction site associated DNA sequencing (ddRAD-seq), 125
Double-strand breaks (DSBs), 178
Drought stress, 328
Drought-stressed region, 52
Durum wheat (*Triticum turgidum* L.), 167

E
Echinochloa esculenta genotype (PRJ 1), 400, 401
Echinochloa frumentacea L., 392
Effective population size, 80
Efficacy and power, GS
EBV, 73
effective population size, 80
gain per unit time, 73
GEBV, 73
heritability, 80
kinship, 81
marker type and density, 80
phenotypic selection, 73
plant species, 74–79
Eleusine, *see* Finger millet
Eleusine coracana, 367, 369
Eleusine indica, 366
archaeological finding, 367
charred debris, 366
diploid species, 367
E. coracana, 366
hybridization pattern, 386
perception, 369
Embryo culture, 9
Embryo production, 195
Embryo rescue, 38, 204
Embryoids, 4
Engineered green fluorescent protein (EGFP), 274
Environment data
crop management, 100
effects, 99
factors, 100
GEI, 100
ideotypes, 100
phenotype plasticity, 99
plant phenotyping, 100
Environmental pollutant, 298
Envirotyping, 100
Estimated breeding value (EBV), 73
EST-simple sequence repeats (EST-SSRs), 401
Ethyl methanesulphonate (EMS), 131
Evolutionary game theory (EGT), 112
Expressed sequence tags (EST), 124, 385, 400, 401

F
FAIRsharing web portal, 99
Fatty acids, 66
F-box protein, 223
F-box-containing protein, 222
Feed and food, barley
antioxidants and phytochemicals
anthocyanins, 320
phenolics, 320
tocopherols/vitamin E, 321
zeaxanthin and lutein, 321
cholesterol-free/low fat content, 319
enzymes, 317
health benefits, 319
livestock feed, 318, 319
malting/brewing, 317, 318
nutritive esteem, 316
vitamins and minerals, 319, 320

Fertile plants (FP), 279
FHG-2 extraction solution, 199, 200
Field evaluation, 251
Finger millet
  botany
    diploids, 368
    flowers, 368
    genus, 368
    leaves, 368
    ovary, 369
    panicle/earhead, 368
    seed, 369
    species, 368
  breeding (*see* Breeding, finger millet)
  cytogenetics, 369
  distribution, 367, 368
  economic importance, 366
  genetic resources
    accessions, 373
    core sets, 374
    inter-species, 373
    NBPGR, 371, 373
    wealth/treasure, 370
  genetics
    qualitative traits, 374, 375
  genomics-assisted breeding, 385, 386
  GS, 386
  nomenclature, 365
  origin, 366, 367
  varieties, 379–383
Flanking genes, 52
Flower buds, 4
Former Project Coordinator of All India Coordinated Small Millets Improvement Project (AICSMIP), 373
Four-dimensional (4D) profile, 113
F_2 recurrent mass GS, 63, 64
F_3 recurrent GS, 64, 65
F_4 recurrent GS, 65
F_7 recurrent GS, 65
Freezing stress
  H^+-ATPase activity, 326
  osmoprotectants, 327
  PM, 326
  radiation frost, 325
  ribosomal RNA, 325
  ROS/AOS, 326
  RuBisCO, 325
  soil salinity, 325, 326
  tolerance, 325
Functional genes, 220, 221
Functional nucleotide polymorphism (FNP), 224
fungal diseases, 384
Fusarium head blight (FHB), 207
Fuzzy c-means (FCM), 111

G

Gauss RBF kernel, 68
GBLUP linear model, 71
GE tool-mediated allele replacement, 229
Gene Engineering, 7
Gene expression studies, 298
Gene pyramiding, 66
Gene/allele-specific markers, 223, 224
General combining ability (GCA), 417
Genetic estimation and prediction, 60
Genetic gain, 59, 60
Genetic male (GMS) sterility, 421
Genetic mapping, 15, 16
Genetic variability, 167
Genome editing, 13, 14
Genome editing system, 427, 428
Genome-wide association studies (GWAS), 92, 294, 402
  high-resolution mapping, 127
  LD, 127, 338
  mapping population, 127
  MLM, 128, 129
  qhir1, 264
  single-locus models, 129
  traits, 128
Genome-wide markers, 57, 59
Genomic best linear unbiased prediction (GBLUP), 112
Genomic estimated breeding values (GEBVs), 54, 57, 60, 63–65, 68, 73, 80–82, 132, 133, 404, 425
Genomic prediction
  accuracy, 71
Genomic selection (GS), 12, 90, 92, 104, 132, 133, 404, 414, 425, 426
  advantages, 52, 53, 57, 81
  bioinformatics and genomics tools, 61–63
  breeding scheme (*see* Breeding scheme, GS)
  development, 57, 58
  DH lines, 52
  disadvantages, 81, 82
  efficacy and power, 73–81
  Flanking genes, 52
  GEBV (*see* Genomic estimated breeding values (GEBVs))
  genetic estimation and prediction, 60
  genetic gain, 59, 60
  genome-wide marker, 57

  high-density marker platform, 53
  MAS (*see* Marker-assisted selection (MAS))
  NGS, 54
  non-phenotyped individuals, 57
  population, 53
  quantitative traits, 57
  rationale, 53
  statistical concept, 71–73
  statistical model, 54 (*see also* GS model)
  strategy, 53
  training population, 57
Genomics and biotechnology tools, 227
Genomics-assisted breeding (GAB), 385, 386
  EST, 400
  EST-SSRs, 401
  genome-wide markers, 400
  GWAS, 402
  hot water emasculation, 402
  low-cost genome sequencing, 402
  microsatellite markers, 401
  reference genome, 400
  well-saturated genetic maps, 402
Genotype dependency, 246
Genotype-environment interactions (GEI), 96, 99, 100, 104
Genotype file formats, 95, 96
Genotypic analysis, 104, 106
Genotyping, 227
Genotyping by sequencing (GBS), 18, 125, 424
Germplasm unit (GPU), 376, 378
GFP-tailswap, 177
Global environment effect, 52
Global position system-real-time kinematic, 54
Glycine max, 66
Green fluorescent protein-tagged (GFP), 177
Green plant regeneration, 235, 239
  androgenic response, 236
  Cold pre-treatment, 238
  gametophytic pathway, 238
  genotypes, 235
  microspores, 237
  photoperiodism nature and light, 239
  physiological growth conditions, 236
  pre-treatment, 238
  pre-treatments, 238
  temperature, 240
  time consuming, 237
Green revolution, 90
Green Revolution rice, 218
Green shoot regeneration, 236, 245
Greenhouse, 32, 33, 41, 42, 44
Growth chambers, 32, 33, 36, 37, 39, 42, 44
GS model
  accuracy of genomic prediction, 71
  Bayesian model, 70
  BLUE, 70
  BLUP, 70
  LS, 69
  parametric method, 71
  phenotype data, 54
GS3 gene encoding, 221

H
Haemocytometer, 199
Haploid breeding method, 337, 340
Haploid embryos, 171
Haploid identification
  automation, 286
  genetic markers, 269–274
  haploids and diploids, 274–276
  HIR, 266
  marker traits, 267
  R1-nj marker, 267
Haploid induction, 247
Haploid induction rate (HIR), 261
  backcrossing, 266
  inducer × inducer/inducer × noninducer, 267
  markers, 266
  MAS, 268
  natural selection, 268
  phenotypes, 267
  qhir1, 268
  tester/inducer × tester interaction, 267
  traits, 268, 269
Haploid male fertility, 280
Haploid plant formation
  cytoplasmic substitution, 141
  DH (*see* Doubled haploid (DH))
  induction methods, 140
  interspecific, 140
  microspore culture, 141
  T. monococcum L., 140
  wide crossing, 140
  x-ray effect, 140
Haploid seedlings, 274
Haploids and diploids, 274, 279
  efficiency and accuracy, 276
  flow cytometry, 275
  R1-nj, 276
  seedling traits, 274, 275
  seminal roots, 274
  stomatal sizes, 275
  variations, 274

Haploids and diploids (*cont.*)
  vegetative and reproductive growth, 276
Harvest index (HI), 225
HarvEST:Barley, 342
Heritability, 80
Heterofertilization, 263, 265
High-throughput genotyping (HTG), 18, 19, 53, 68
  EST sequencing, 124
  GBS, 125
  genome resequencing-based, 125
  NGS, 125
  SNP, 124
  WGR, 125
High-throughput phenotyping (HTP), 17, 18, 90
  flexible and cost-effective, 126
  gene discovery, 126
  noninvasive image analysis, 126
  remote sensing technologies, 126
  traditional, 126
  trait dissection, 126
High-yield traits/genes, 225
High-yielding genes, 226
Histone deacetylase inhibitors (HDACi), 197
Histone fold domain (HFD), 177, 178
Homology directed repair pathway (HDR), 90
Homology-directed repair (HDR), 13, 302
Homozygosity, 284
Hordearii, 309
Hordeum chromosomes, 176
Hordeum vulgare, 310
Hull-less barley
  cultivars, 322
  distribution, 321
  hereditary assets, 321
  nutritional constituents, 321
  nutritious human sustenance, 322
  waxy and normal, 321
Hybrid development process, 301
Hybrid zygotes, 171
Hybridization, 397
2-Hydroxynicotinic acid (2-HNA), 147

I
Imperata cylindrica, 172, 173
In vitro haploid induction
  chromosome doubling, 260
  DH production pipelines, 260
  MHI, 261, 262
  PHI, 260
  TAILs, 262
In vivo haploid induction, 259, 260
Indaf varieties, 376
Indian Council of Agricultural Research (ICAR), 413
Indian Institute of Millets Research (ICAR-IIMR), 413
Indica rice, 234
Indole acetic acid (IAA), 244
Inducer-mediated genome editing (IMGE) approach, 7
Infrared gas analyzers, 54
Infrared thermography, 54
Inorganic nitrogen, 241
International Crops Research Institute for Semi-Arid Tropics (ICRISAT), 371, 373
International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), 393
International Maize and Wheat Improvement Center (CIMMYT), 150, 259
International Rice Research Institute (IRRI), 217
International Sorghum and Millet (INTSORMIL), 414
Isolated microspore culture, 4

K
Kinship, 81
Kompetitive Allele-Specific PCR (KASP) assay, 62

L
Least absolute shrinkage and selection operator (LASSO), 132
Least square (LS), 67, 69
LeNet architecture, 109
Light-emitting diodes (LEDs), 11, 32
Linkage disequilibrium (LD), 92, 127, 338
Linkage mapping, 53
Liquid chromatography-tandem mass spectrometry (LC-MS/MS), 97
Livestock feed, 318, 319
Lodging, 329, 330
Low-density lipoprotein (LDL), 319

M
Machine learning (ML), 68, 104, 106
Madayyanagiri, 375
Maize
  biofortification, 293
  calories in human diets, 293
  phytase, 299

QTLs, 294
Maize (*Zea mays* L.)
breeders and geneticists, 258
CIMMYT, 259
DH line (*see* DH line production process)
doubling haploid genome, 276–280
extensive evaluation, 258
food security, 257
hybrid breeding programs, 257
in vivo induced maternal haploids identification, 269–276
yields, 257
Maize breeding, DH lines
conventional inbred lines, 284
development, 283
genetic variation, 285
haploid stage, 285
heritabilities, 284
homozygosity, 284
landrace-derived, 285
marker/QTL effects, 284
transgene stacking, 285
Maize method
Bulbosum, 141
chromosome doubling, 145, 146
embryo rescue, 145
embryo size, 142
grain forming/swelling stimulation, 144
Panicoideae, 142
pollination, 142–144
Male sterile-facilitated recurrent selection (MSFRS), 335
Malondialdehyde (MDA), 326
Malting/brewing, 317, 318
Maltose, 241
Marker validation, 66
Marker-assisted backcrossing (MABC), 66, 294
Marker-assisted background selection (MABS), 15
Marker-assisted breeding (MAB), 65, 332, 341
genotypes, 294
methionine, 297
phenotypic selection, 295
phytic acid, 298
QPM genotypes, 296
tryptophan and lysine, 294
Marker-assisted selection (MAS), 14, 52, 55, 56, 92, 132, 168, 182, 268, 316, 386, 404, 414
advantages, 66
Bayesian estimation, 67
BLUP method, 67
gene pyramiding, 66
genetic introgression, 66
high-throughput genotyping, 68
LS method, 67
machine learning, 68
marker-assisted backcrossing, 66
Marker-based selection, 55
Marker-trait association (MTA), 54
Markov chain Monte Carlo (MCMC), 67
Maternal haploid induction (MHI), 261, 262
Mating designs, 122
MATRILINEAL (MTL), 154, 264
Medicago truncatula, 17
Mendelian inheritance, 262
Meta-analysis, 112
Methionine, 297
Micro- and macronutrients, 240
carbon source, 240
cytokinins, 241
growth regulators, 241
nitrogen, 241
Micronutrients, 240
Micropropagation, 7
Minimal Information About Plant Phenotyping Experiments (MIAPPE), 100, 107
Minimum information (MI), 99
Minor allele frequency (MAF), 127
Mixed linear models (MLM), 128
ML algorithms, 109–111
Modern breeding approaches
GAB, 400–402
interspecific hybridization, 399, 400
mutation breeding, 399
Molecular markers, 54
DNA-based, 123
hybridization-based RFLP, 123
PCR-based markers, 123, 124
QTL, 123
Monogastric animals, 318
Morpho-agronomic evaluation, 247
MTL/NLD/ZmPAL1 gene, 264
Multidimensional scaling, 395
Multilayer perceptron (MLP), 104
Multi-parent advanced generation inter-cross (MAGIC), 127
Multi-trait deep learning (MTDL), 112
Multi-trait multi-environment (MTME), 112
Mutation breeding, 399, 428
MutMap, 130, 131

N

National Aeronautics and Space Administration (NASA), 33

National Agricultural Research Systems (NARS), 259
National Bureau of Plant Genetic Resources (NBPGR), 371
National Center for Biotechnology Information (NCBI), 223
Navajo phenotype, 269, 271, 273
Near-infrared (NIR) spectroscopy, 17
Nested association mapping (NAM), 414, 425
Next-generation plant breeding, 61
Next-generation sequencing (NGS), 54, 61, 125
NGS-based BSA
  biparental population, 129
  BSR-seq, 131
  DNA resequencing, 131
  MutMap, 130, 131
  MutMap+, 131
  QTL-seq, 129
  RNA-seq analysis, 131
  SNP index, 129
Nitrogen use efficiency (NUE), 112
Nitrogen, phosphorous and potassium (NPK), 250
NMR-based automated platform, 272
Non-homologous end joining (NHEJ), 90
Noninducer pollen, 265
Non-parametric methods, 71
North and Sub-Saharan Africa (SSA), 309
NRC-Saskatoon, 207
Nuclear magnetic resonance (NMR), 17, 97, 272
Nutrient starvation, 238
Nutrient stress, 330, 331
Nutritional security, 344

O
Opaque2 allele, 296
Open-pollinated varieties (OPVs), 264
Osmolarity, 148
Ovaries/ovary conditioned medium, (OVCM), 148
Ovary culture, 5

P
Panicle/earhead, 368
Panicoideae, 142
Parafilm®, 207, 209
Partitioning of genetic variation, 122
Paternal haploid induction (PHI), 260
Pedigree breeding, 336
Phenolics, 320
Phenotypic analysis
  annotated training data, 109
  CNN-based algorithms, 109
  computer vision-based phenotyping, 109
  LeNet architecture, 109
  MIAPPE, 107
  ML methods, 107
  modelling GEI, 111–112
  regression performance, 109
  SPIKE, 109
  transfer learning, 111
Phenylacetic acid (PAA), 234
Photosynthetically active radiance (PAR), 43, 205
Phytic acid, 298
Phytoene desaturase (PDS), 427
Phytohormones, 234
Plant breeding, 1
  achievements, 89
  advancement, 89, 90
  crop varieties development, 89
Plant breeding innovation
  Breeding 4.0, 92
  data (*see* Data management)
  GWAS and GS, 92
  isolations, 92
  molecular genomic techniques, 92
  mutations, 92
  phases, 90, 91
  speed breeding, 92
Plant growth regulators (PGRs), 241
  auxins, 244
  cytokinin, 244
  NAA-induced calli, 244
  polyamine, 244
Plasma membrane (PM), 326
Polyamine, 244
Polygenic systems, 55
Polymerase chain reaction (PCR), 13, 16
Pre-breeding programmes, 182
Precision breeding, 90
Principal component analysis (PCA), 395
Probabilistic neural network (PNN), 111
Protection of Plant Varieties and Farmers' Rights (PPV&FR), 375
Protein storage vacuoles (PSV), 298
Provitamin A, 296
Purple sheath marker, 273

Q
QPM hybrids, 296, 301
QTL-seq, 129, 131
Quantitative genetics

Darwin's idea of selection, 121
genomic selection, 131–133
high-throughput genotyping, 124–125
HTP, 126
mapping (*see* QTL mapping)
molecular markers, 123–124
regression, 121
traits (*see* Quantitative traits)
Quantitative PCR high-resolution melting (qPCR-HRM), 14
Quantitative trait loci (QTL), 12, 123, 220, 294, 297, 425
chromosome, 300
Fe and Zn, 300
gene identification, 54
haploid induction, 263
identification, 53, 54
mapping, 59
metal transporter genes, 300
ZmZIP5 expression, 300
Quantitative trait locus (QTL) mapping, 66, 92, 234
biparental mapping population, 124
crop improvement programme, 124
GWAS, 127–129
NGS-based BSA, 129–131
phenotypic traits, 123
principle, 123
procedures, 126
Quantitative traits
BSA-based modifications, 129
DNA segments, 123
genetic gains, 122
GWAS, 129
inheritance and linkage phenomenon, 123
MAS, 132
mating designs, 122
Mendelian inheritance, 122
phenotyping procedures, 126, 132
QTL, 123, 124, 130
remote sensing technologies, 126
selection accuracy, 122
speed breeding, 126
statistical tools, 122
Quantitative variation, 121

R

R1-nj marker
anthocyanin inhibitor genes, 270
expression, 271
false positives and false negatives, 271
haploid identification, 269
mechanical sorting, 270
Navajo phenotype, 269
red/purple root coloration, 273
Radial basis function neural network (RBFNN), 68
Random amplification of polymorphic DNA (RAPD), 16, 385
Random forest (RF), 68
Rapid generation advancement (RGA), 37, 41
Reactive oxygen species (ROS), 322, 323
Recombinant inbred lines (RILs), 66, 424, 425
Recurrent neural networks (RNN), 104
Red fluorescent protein (RFP), 178
Red-green-blue (RGB), 17
Red root marker, 272–274, 276
Red-root-marker-based haploid identification, 273
Remote sensing technologies, 126
Research Institute for Bioresources (RIB), 342
Restriction fragment length polymorphism (RFLP), 16, 66, 123
Restriction fragment sequencing (Rest-seq), 125
Restriction site associated DNA sequencing (RAD-seq), 125
Reverse breeding (RB), 12, 13
Rice Annotation Project Database (RAP-DB), 223
Rice production
APO1-overexpressing mutant, 222
DEP1 protein, 221
DNA methylation, 220
genes, 218
genetics, genomics and biotechnology, 218
genotyping, 224
Ghd7 alleles, 221
global population, 217
GS5 gene, 222
GW2 gene encoding, 223
IPA1 allele, 221
IR8, 218
NAL1 gene, 222
NOG1, 223
OsCKX2 coding sequence, 220
OsSPL16, 221
SPIKE allele, 222
TGW6, 222
traditional technologies, 217
yield-related genes, 218
Rice-breeding efforts, 233
Ridge regression best linear unbiased predictors (rrBLUP), 132
RKHS model, 71
RNA interference (RNAi), 426
Root induction, 249

S
Seed production, DH lines
  biodegradable jiffy pots, 281
  chromosomal doubling treatments, 280
  colchicine-treated seedlings, 280
  D_0 nursery, 282
  fertilizers, 282
  field conditions, 281
  haploid tassels, 282
  insecticides and fungicides, 282
  planting haploids, 281
  soluble fertilizers, 280
  tassel bags, 282
Seedling immersion method, 277
Segregation-distortion-based QTL mapping, 264
Selection with markers and advanced recombination technology (SMART), 341
Self-pollination, D_0 plant, 282, 283
Semi-arid region, 52
Sequence tagged sites (STS), 66
Sequence-dependent simple sequence repeat (SSR), 385
Sequence-specific nucleases (SSNs), 13
Serine acetyltransferase (SAT), 297
Shrunken endosperm, 266
Shuttle breeding, 11
Silver nitrate ($AgNO_3$), 245
Simple sequence repeats (SSRs), 16, 66, 340
Single nucleotide polymorphisms (SNPs), 12, 16, 18, 53, 57, 66, 92, 124, 395
Single seed descent (SSD), 11, 16, 17, 44, 336
Skim-based genotyping by sequencing (Skim-GBS), 125
Small- and medium-enterprise (SME), 259
SNP index, 129
Solid media, 246
Somaclonal variation, 8, 9
Sorghum, 37, 38
Sorghum improvement
  adoption of hybrids, 413
  agriculture, 411
  apomixis, 428, 429
  arid and semi-arid regions, 411
  bicolor types, 412
  breeding strategies
    association mapping, 424, 425
    genome editing system, 427, 428
    genomics, 423, 424
    GS, 425, 426
    QTL mapping studies, 423, 424
    transgenic approach, 426, 427
  brown midrib (bmr), 421
  crop improvement, 411
  energy crop, 429
  gene editing, 429
  genetic improvement, 414
  germplasm, 412, 413
  harnessing natural variability, 414–416
  hybrid breeding, 416, 417, 419
  male sterility, 421
  mass selection, 412
  maturity, 419, 420
  mutation breeding, 428
  plant breeding, 429
  plant height, 419, 420
  progenies and multi-location evaluation, 412
  tools and techniques, 414
  transgenics, 429
  types, 412
  Working Groups, 412
Sorghum propinquum, 38
Specific combining ability (SCA), 417
Specific leaf area (SLA), 112
Specific-locus amplified fragment sequencing (SLAF-seq), 125
Speed breeding, 2, 10, 11, 92, 126
  accelerated breeding, 202
  cereals
    adult plant resistance, 36
    air conditioner, 35
    application, 36
    biotron breeding system, 36
    controlled environment chamber, 35
    controlled environmental condition, 33
    embryo rescue method, 38
    growth of plants, 35
    hexaploid, 36
    phenotyping multi-traits, 36
    RGA, 37
    roots and shoots, 38
    seeds, 38
    sorghum, 37, 38
    temperature-controlled glasshouse, 35
    tetraploid, 36
    wheat and barley, 36
  climate change, 31
  conventional breeding programmes, 32
  conventional methods, 31
  conventional plant growth conditions, 201, 203
  crop improvement programmes, 32
  crops
    Apple (*Malus x domestica* Borkh.) breeding, 45
    embryos, 42

environment-controlled growth chambers, 39
fruit crops, 45
genomics-assisted breeding, 46
grain amaranth, 46
greenhouse technology, 41
harvesting, 41
hydroponics and intermediate methods, 44
immature pods, 39
in vitro and in vivo strategy, 42
in vitro methods, 43
mature faba bean seeds, 44
oilseeds, 39
plant proteins, 40
protein-rich seed legumes, 40
protocol, 39
radio spectrometer, 44
RGA, 41
seed treatment, 40
sesame, 40
soybean seed maturation studies, 39
subtropical and temperate countries, 39
sunflower, 40
temperature, 41
tissue culture methods, 44
vegetable breeding, 46
crop yields, 31
crossing, 203, 204
embryo rescue, 204
genes of interest, 203
genotypic and phenotypic selection, 207
germination, 203
history, 32, 33
influence of environment, 31
lighting, 205
methods and application, 33–35
phenotyping, 32
plant breeders, 31
protocol
crossing, 210
donors embryo rescue, 208, 209
donors plants growth, 209, 210
recurrent parents growth, 207, 208
single-seed-descent protocols, 207
spring wheat maturation, 203
stressors, 206
vernalization, 204, 205
winter wheat growth, 206
Spike culture, 144
Spontaneous chromosomal doubling
AER, 279
FP, 279
haploid male fertility, 279, 280
heritabilities, 280
mechanisms, 280
Spontaneous genome doubling, 249
Standardized ontologies, 101
Superoxide dismutase (SOD), 320, 326
Support vector machine (SVM), 68, 104, 106, 107, 109

T

Tassel fertility, 282, 283
Temperature stress
canopy system, 324
climate change, 323
glaucousness, 324
heat, 323
normal temperature conditions, 324
photosynthetic machinery, 324
premature progression, 324
ranges, 323
yield losses, 324
Test population (TSP), 53
Tetraploid finger millet, 386
The European Infrastructure for Multi-scale Plant Phenomics and Simulation (EMPHASIS) project, 99
Threshold genomic best linear unbiased prediction (TGBLUP), 104
Tissue culture incubation, 239
Tissue culture media, 240
Toxic aluminum (Al), 327
Training population (TRP), 53
Transcription activator like effector nucleases (TALENs), 13, 178
Transfer learning, 111
Transgenic approach, 298, 426, 427
Transgenic breeding, 9, 10
Trichoderma harzianum, 427
Trichostatin (TSA), 197
Triticeae species, 141
Tropically adapted haploid inducer lines (TAILs), 262
Tryptophan and lysine, 294
Twin plants, 263

U

Unmanned aerial vehicle (UAV), 17

V

Vernalization, 204–206
Vivek QPM 9, 295

W
Waterlogging, 329
Water-soluble carbohydrates (WSC), 324
Wheat, 32, 33, 35, 36
Wheat breeding
- CEN3 mutations, 153
- CENH3, 154
- CIMMYT, 150
- conventional breeding, 150
- DH-derived wheat varieties, 150
- Glossa, 150
- Jinghua #1, 149
- junk plants, 153
- MATRILINEAL, 154
- programs, 150

Wheat × *Imperata cylindrica* system
- anthesis, 175
- chromosome elimination, 173
- comparative efficiencies, 173, 174
- cytological investigation, 173
- haploid formation efficiency, 175
- haploid induction, 175
- hybrid seed, 175, 176
- pollen source, 173
- synchronization, 173

Wheat × maize system
- DH, 171
- Durum/macaroni, 171
- embryo formation, 171
- genotype non-specificity, 171
- haploid induction efficiency, 171
- haploid plantlet regeneration, 171
- non-synchronization, 172, 173

Whole genome resequencing (WGR), 125
Whole genome sequencing (WGS) methods, 223
Wide hybridization
- *bulbosum method*, 170
- wheat × *Imperata cylindrica* system, 173–176
- wheat × maize system, 171–173

Wild barnyard millet (*Echinochloa colona*), 391
Winter wheat
- advantages, 191
- androgenesis, 193–201
- breeding objectives, 396, 397
- breeding programs, 191
- DH breeding program, 194–195, 201, 202
- DH cultivars, 195, 202
- doubled haploidy technology, 192
- importance, 191
- speed breeding (*see* Speed breeding)
- wheat × maize method, 193

Y
Yellow mosaic viruses, 340
Yield-enhancing genes, 224
Yield response models, 112
Yield-related genes, 219

Z
Zea mays, 66
Zeaxanthin and lutein, 321
Zinc finger nucleases (ZFNs), 13, 178, 228, 302
ZmDMP gene encoding, 264
ZmZIP5, 300