Hee-Jong Koh · Suk-Yoon Kwon
Michael Thomson *Editors*

Current Technologies in Plant Molecular Breeding

A Guide Book of Plant Molecular Breeding for Researchers

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Preface

Plant breeding, the science for plant genetic improvement, made great progress in the twentieth century with the rediscovery of Mendelian genetic principles in 1900. Most of the traditional breeding methods were established before the 1960s leading to the development of high-yielding varieties in cereal crops which brought the "Green Revolution" during the 1960s–1980s. Recent progress in biotechnology and genomics has expanded the breeders' horizon providing a molecular platform on the traditional plant breeding, which is now known as "plant molecular breeding." Under a new paradigm of plant breeding in the twenty-first century, breeders try to create new variation through the direct manipulation of target genes instead of phenotype-based trait selection. Genetic resources are extended to the unrelated species because transgenic technologies break through the sexual limit for gene transfer. In addition, selection and genetic fixation in the progeny can be performed by monitoring of genes and genomics information by which breeders can develop new varieties precisely and quickly.

Although diverse technologies for molecular breeding have been developed and applied individually for plant genetic improvement, the common use in routine breeding programs seems to be limited probably due to the complexity and incomplete understanding of the technologies. This book is intended to provide a guide for researchers or graduate students involved in plant molecular breeding by describing principles and application of recently developed technologies with actual case studies for practical use.

This book is organized in nine chapters. In Chap. 1, a brief history and perspectives of plant breeding are presented, including the directions of future development of breeding methods. In Chap. 2, the basics on genetic analysis of agronomic traits are described, including how to construct molecular maps and how to develop DNA markers. In Chap. 3, methods of detecting QTLs are illustrated, while in Chap. 4, the application of molecular markers in actual plant breeding is described in detail with case studies. In Chap. 5, genome sequencing and how to analyze the association between sequencing data and phenotype are introduced, including the epigenome and its possible application to plant breeding. In Chap. 6, genome-wide association studies are explained so that researchers can analyze the data following

the manual including the introduction of software for analysis of population structure. In Chap. 7, methods for mutation screening and targeted mutagenesis are described. In Chap. 8, how to isolate the genes of interest and how to analyze the gene function are presented with case studies. In Chap. 9, the basics of gene transfer in major crops and the procedures for commercialization of GM crops are explained.

We attempted to cover most of the molecular tools applicable in plant breeding; however, due to the limitation of the book volume, we had to skip some skills that are still under development. Therefore, in this book, only key technologies which are currently used in plant breeding are mentioned. Since technologies per se are being advanced, we may add newly emerging ones with a chance given later. We hope this book would be a valuable reference for plant molecular breeders and, in addition, will become a cornerstone for the development of new technologies in plant molecular breeding for the future.

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Chapter 1
Brief History and Perspectives on Plant Breeding

Joohyun Lee, Joong Hyoun Chin, Sang Nag Ahn, and Hee-Jong Koh

Abstract Following the rediscovery of Mendel's principles of heredity in 1900, plant breeding has made tremendous progress in developing diverse methodologies to create and select variation by using genetic principles. Since the beginning of the twenty-first century, plant breeding has been systematized with state-of-the-art technologies aided by transgenic and genomics approaches. In the future, breeders will be able to assemble desirable alleles or genes into promising varieties with optimized performance using an approach that integrates scientific fields. Recent concerns about global warming, abnormal weather patterns, and unfavorable environments have pushed breeders to speed up the breeding process. In this chapter, the history of plant breeding, methods for creating variation, selection and generation advance strategies, and challenges and perspectives are briefly reviewed and discussed.

1.1 Brief History of Plant Breeding

Humans began managing wild plants in fields about 12,000 years ago; since then, plants have undergone a series of adaptive changes in production and food-associated traits, called domestication or adaptation syndromes. Early human farmers acted as

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breeders by cultivating and selecting better plants or seeds in anticipation of better performance in the next season (Hancock 2004).

Systematic breeding was not performed until 200 years ago. The existence of sex in plants was first recognized by Rudolf Camerarius in 1694. The first manual crossing was performed in 1717 by Thomas Fairchild, who developed the first artificial hybrid by crossing carnation (*Dianthus caryophyllus*) and sweet William (*Dianthus barbatus*). In the early nineteenth century, Patrick Shirreff developed new varieties of oat and wheat via selection and crossbreeding and are now regarded as the first cereal breeder. Knowledge began to accumulate on plant biology, such as cells, sexual reproduction, and chromosomes. However, because the gene concept was not formulated at that time, plant breeding was performed empirically, without a theoretical background. In 1865, Gregor Mendel published 'Experiments on plant hybridization', his genetic experiment with garden pea that is now the foundation for modern genetics and breeding. However, his hypothesis on plant genetics was not widely accepted scientifically for 40 years (Stoskopf 1993).

After Mendelian genetic law was confirmed in 1900, breeders began to develop new varieties based on these genetic principles. Despite the short history of scientific plant breeding, conventional breeding methods have dramatically improved crop yields in corn, rice, wheat, and other crops. The Food and Agriculture Organization of the United Nations (FAO) reported that in the two decades from 1965 to 1985, crop yield increased 56 % worldwide, whereas from 1985 to 2005, only a 28 % increase was recorded. The rapid yield improvements from 1965 to 1985, called the "Green Revolution", resulted from the introduction of genetically-improved varieties, treatment with fertilizers and pesticides, improved irrigation systems, and mechanization of agriculture. We are now facing new challenges to a stable food supply because of global warming, abnormal weather patterns, water shortages, increased demands on crops for bio-fuel, reduced arable land, and mounting population pressure. The global human population is expected to increase by 1 billion people every 14 years and to reach 10 billion within 25–30 years; stable food supply will require 70–100 % more crop production by then. Moreover, this goal must be achieved under unfavorable environmental conditions (Foley et al. 2011). To overcome these challenges, breeders should use all possible technologies to improve yield. Thus advanced biotechnology which can create new genetic variation, and the molecular technology for selecting superior genotypes will be essential in breeding programs to increase crop yield and provide a stable and sustainable food supply.

Plant breeding comprises two main steps: creating or expanding new variation and selecting and fixing desirable genotypes in the progeny (Fig. 1.1). Variation that meets breeders' goals should primarily exist in the germplasm. In the history of plant breeding, methods for creating useful variation, such as artificial crossing, induced mutation, and polyploidization (chromosome manipulation), were used relatively early. Once tissue-culture techniques were established, cell fusion, tissue culture, and inter-specific hybridization were added to the repertoire of methods. Recently, transgenic technology for introducing foreign genes into crops has

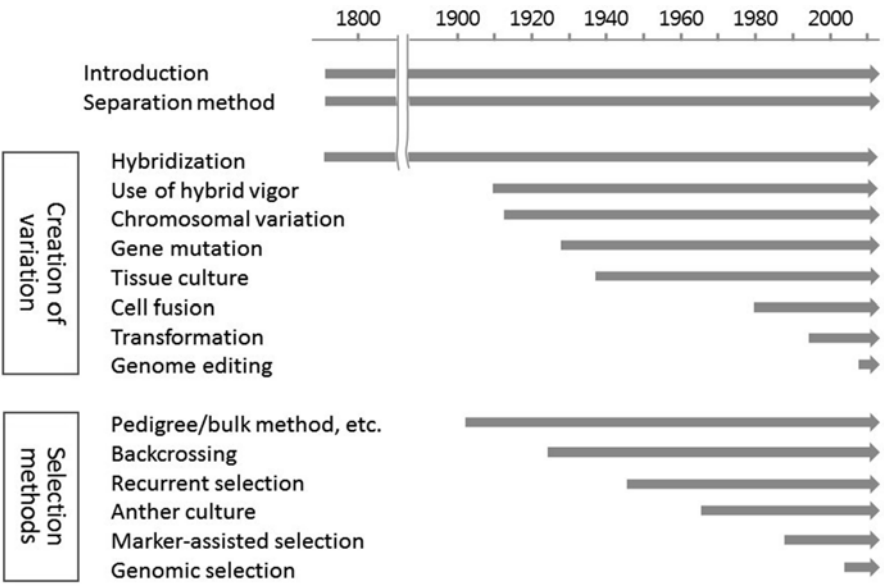

Fig. 1.1 Brief history of plant breeding methods

become available, and targeted mutation and genome editing technologies are under development for crop breeding. Lusser et al. (2011) listed the new plant breeding techniques with a focus on the creation of novel variation.

Even when useful variation exists, without an appropriate method to detect it and to select progeny that contain the target genotypes, breeding goals cannot be accomplished. Thus, the selection method is a critical determinant for successful plant breeding. Pedigree or bulk selection, backcross breeding, recurrent selection, and anther culture are methods for selecting and propagating progeny to fix a desirable trait for the next generation. Several types of molecular marker techniques are available to evaluate selection efficiency and eventually improve breeding efficiency. With accumulated genome sequence information and next-generation sequencing (NGS) techniques for high throughput sequencing, pioneering efforts for sequence-based genomics election have been initiated.

The most important milestone in plant breeding was the Green Revolution, the drastic increase in crop productivity through the development of high-yielding semi-dwarf varieties of wheat and rice. Norman Borlaug, Nobel laureate and father of the Green Revolution indicated that the main reasons for the success of these semi-dwarf varieties were wide adaptation, short plant height, high responsiveness to fertilizer, and disease resistance (1971). The International Rice Research Institute (IRRI) team developed a semi-dwarf rice variety, IR8, in 1962. IR8 had stiff straw to resist lodging and was insensitive to photoperiod, making it widely adaptable. The increase in food production led to more crops being grown per unit of land and with similar effort to that before the Green Revolution. Thus, production costs were reduced, eventually resulting in cheaper food prices at market. Also, this high

productivity benefitted the environment because fewer natural areas were needed to cultivate crops. From 1961 to 2008, as the human population increased by 100 % and food production rose by 150 %, the amount of forests and natural land converted to farmland increased by only 10 % (FAO 2014).

1.2 Methods for Creating Variation

1.2.1 Hybridization

Artificial hybridization has long been a main method for creating new varieties through recombination between parents. In general, hybridization achieves breeding objectives in two ways. One is combination breeding, which combines beneficial traits from parents into desirable genotypes. For example, when one parent has disease resistance and another has insect resistance, a breeder can combine both traits in a line by crossing and progeny selection. The other method is transgression breeding: the selection of transgressive segregants in the progeny that outperform parents. This phenomenon usually occurs with quantitative traits, because several alleles at different loci for a certain trait accumulate through gene recombination. For example, when breeders attempt to develop extremely early-flowering varieties, they cross early varieties to generate transgressive segregants that flower extremely early as a result of the accumulation of alleles for earliness. Transgressive segregation is detected more frequently in progeny from wide crosses than crosses between parents of similar genetic makeup.

The principle underlying artificial hybridization is the recombination or reassembly of genes in which additive effects and epistasis among the reassembled genes improve traits in the progeny, as compared with their parents. However, using a wide range of germplasm in hybridization breeding remains difficult because artificial hybridization can only occur between cross-compatible germplasm.

1.2.2 Induced Mutation

After the first discovery by Muller and Dippel (1926) that X-rays could cause mutations in *Drosophila*, induced mutants in tobacco, *Datura*, and corn were quickly reported (1927–1928). X-rays and chemical mutagens were used not only in plant science for gene identification but also in plant breeding to develop new varieties. In the 1940s, German scientists introduced *Arabidopsis thaliana* as a model plant in mutation research. Although many plants required screening to select mutant phenotypes, and a fairly long time was needed to develop a new variety from the selected mutants, mutation breeding initiated in the 1950s was quite successful.

Most mutant varieties have been developed in China, India, and developing countries. Mutant varieties of fruits, such as apple, grape, peach, pear, and papaya,

were mostly developed by private companies. According to the mutant variety database of the Joint FAO/IAEA Program (http://mvgs.iaea.org/Search.aspx), more than 3,000 mutant varieties have been developed worldwide. Mutation breeding by physical or chemical mutagens is not as popular as the recently developed method of transformation, but it has been widely used worldwide. Recently, new mutation methods have been introduced, such as space mutation and ion-beam mutation.

1.2.3 Chromosome Manipulation

Autopolyploids, which are developed by duplicating a genome, have been successfully adopted in vegetatively propagated and ornamental crops. Among grain crops, only tetraploid rye in Eastern Europe has been commercialized. Amphidiploids, which have pairs of more than two different genome sets and thus exhibit normal fertility despite their polyploidy, are expected to be useful, even in grain crops. The classic example of an artificial amphidiploid crop is triticale, which is hexaploid or octaploid and considered the first manmade crop. Triticale was developed by the doubling chromosomes of an F_1 hybrid between tetraploid or hexaploid wheat (*Triticum* spp.) and rye (*Secale cereale*). In addition, chromosomal aneuploidy and structural variations cause abnormal morphology and thus are highly applicable to breeding new varieties of vegetatively propagated ornamental plants.

1.2.4 F_1 Hybrids

F_1 hybrids with hybrid vigor have been a main source of new varieties in cross-pollinated crops, such as maize, since the 1940s, and they are widely used even in self-pollinated crops today. Currently, most commercial seeds for maize, cabbage, radish, and pepper sold by commercial seed companies are F_1 hybrid varieties. Hybrid vigor is the phenomenon that the F_1 hybrids perform better than both parents. Although the mechanism of hybrid vigor is not fully understood, hypothesized explanations include dominance and overdominance, epistatic interactions, and epigenetic control (Chen 2013).

To develop superior F_1 hybrid varieties, breeding lines with high general combining ability and parental combinations having high specific combining ability should be chosen. Then, a method for large-scale F_1 seed production should be established using genetic tools such as cytoplasmic or genic male sterility, self-incompatibility, monoecy, dioecy, or gametocide (chemical pollen suppressor). Because of the costs of hybrid seed production, hybrid vigor must be high enough to compensate for the expenses. In the United States, maize yield was increased from 1.8 to 7.8 t/ha (≈430 %) by cultivation of F_1 hybrids. F_1 hybrid varieties account for 65 % of the worldwide cultivated area for maize, 60 % for sunflower, 48 % for sorghum, and 12 % for rice. Most commercial varieties of Brassicaceae and Cucurbitaceae are F_1 hybrids.

1.2.5 Transgenic Approach

Transgenic crops can be developed by artificially introducing genes. This technology has been the most rapidly applied biotechnology in agronomic history. From 1996 to 2013, the area cultivated with genetically modified (GM) organisms increased from 1.7 to 175 Mha, roughly a 103-fold increase. Recently, biotech crops with stacked traits, involving the introduction of more than two genes, are gaining popularity and were planted in 47.1 Mha in 2013 (27 % of total GM crop area). The market share for GM crops in 2013 was 79 % for soybean, 70 % for cotton, 32 % for maize, and 24 % for rape (James 2013).

From the viewpoint of plant breeding, transformation can introduce novel variations originated from other species. Today, most cultivated transgenic crops have herbicide or insect resistance obtained owing to a few modified genes. Although resistances to weeds and insects are not directly associated with yield, weeds or insects can reduce yield. In addition, biotic stresses, such as viruses, bacteria, fungi, and nematodes, can cause more serious yield losses. A few transgenic crops with virus resistance are available in potato and papaya, but transgenic virus resistance in cereal crops is still years from the market. Moreover, resistance to bacteria, fungi, and nematode is much more difficult to develop transgenically than virus resistance. In particular, for broad-spectrum resistance to fungi, several genes must be introduced, a very challenging task. In this case, conventional breeding strategies may be more efficient.

Abiotic stresses, such as salt, drought, cold, and high temperatures, threaten stable production, and therefore a large amount of transgenic crop research has been conducted around the world; however, progress has been slow. A main reason is that the precise mechanisms of these abiotic stresses are not known. Another problem is that crops generally experience varying environmental conditions, so they can be affected by multiple abiotic stresses at the same time, which harm crops more seriously than individual abiotic stresses. Recent research has shown that when several abiotic stresses are applied, plants respond quite differently than to the individual stresses. Therefore, to develop abiotic stress-resistant transgenic crops, various stress responses must be considered together.

Developing salt-tolerant crops by conventional breeding has progressed slowly, because many quantitative genes are involved and the mechanism is quite complex. Thus, salt-tolerant varieties are unlikely to be developed by transgenic approaches alone. Drought resistance, in which responses to various abiotic stresses are associated, is also important. GM maize with a 6–10 % yield increase under drought conditions was developed by Monsanto in 2013 using a cold shock gene (*cspB*) from *Bacillus subtilis* (http//Monsanto.mediaroom.com).

The next target for GM crops will be improving quality. Attempts have begun to modify the composition of fatty acids in rape seed to produce bio-diesel, and nutrient oils to prevent heart disease. Golden rice, which biosynthesizes beta-carotene, a precursor of vitamin A, was developed to prevent deficiencies in dietary vitamin A. Additionally, GM crops, such as GM banana, tomato, or carrot that express antigens for medical vaccines are under development.

1.3 Selection and Generation Advancement

1.3.1 Simple Phenotypic Selection

In the twentieth century, selection was based mainly on the phenotypic evaluation of target traits. Semi-dwarfism for high yields in rice and wheat is a typical success story. However, because of a paucity of analytical tools for phenotyping and the complicated nature of traits, particularly quantitative ones, simple phenotypic selection has seemingly stagnated in most breeding programs.

Target phenotypes can be divided into qualitative and quantitative traits. Qualitative traits, which are controlled by one or a few genes with genotypes that are easily distinguishable by phenotype, respond readily to simple phenotypic selection. In contrast, quantitative traits, which involve multiple genes, have low heritabilities and are difficult to select phenotypically. Moreover, the effect of selection should be lower in early breeding generations because of dominance effects in the population. Although index selection may be a good alternative, it is rarely applied to field selection on a large scale because of the high cost and effort. Therefore, breeders have empirically selected desirable plants in segregating populations or lines using a truncation selection method, which resulted in limited genetic gain after selection.

1.3.2 Recurrent Selection

Recurrent selection is a method to develop promising populations or lines by pyramiding genes involved in target traits. In the beginning, it was applied to cross-pollinated crops for population improvement and breeding inbred lines. Recently, it has been widely used even in self-pollinated crops to develop useful variations using genic-male sterility in every generation (Zhao et al. 2007). In rice, a MAGIC (Multi-parent Advanced Generation Inter-Cross populations) population, developed by recurrent hybridization among progeny derived from crosses among diverse germplasm, was used to accumulate genes that improved target traits (Bandillo et al. 2013). However, a limitation of phenotypic recurrent selection is that only dominant genes are chosen for the next round of hybridization, because recessive genes are hidden and may be lost in segregating populations. Marker-assisted selection (MAS) should be incorporated for greater success.

1.3.3 Marker-Assisted Selection (MAS)

MAS applies molecular-marker technology to conventional breeding. DNA markers are commonly used in MAS. In general breeding, phenotype evaluation and selection of desirable phenotypes in the progeny are conducted repeatedly from F_2. Molecular markers can be used to reduce the time, cost, and labor of the process. In

addition, molecular markers can be used in various breeding fields: genetic diversity analysis, genotype identification via DNA fingerprinting, genetic mapping of qualitative and quantitative traits, and MAS.

The most representative application of DNA markers in plant breeding is MAS, which is superior to phenotypic selection. MAS can be performed in the early seedling stage and provides high confidence in selection. For traits that are difficult to evaluate such as disease resistance, which requires a special facility to grow pathogens and an inoculating system, MAS will be very effective. MAS can also select recessive traits hidden in heterozygous genotypes. MABC (marker-assisted backcrossing) is a simple application of MAS that is widely used in breeding programs. One or a few targeted quantitative trait loci (QTLs) can be easily introgressed into elite lines through MABC. In conventional breeding, during backcrossing, the progeny are selected based on the target phenotype, whereas in MABC, the selection is based on the DNA marker genotypes. In general, both foreground and background selection are conducted at the same time. Foreground selection confirms the presence of the desired allele from the donor parents until the final backcross, whereas background selection eliminates unwanted genomic introgression from the donor parent. Thus, in background selection, markers distributed across the whole genome are required. A well-known example is IRRI's efforts to develop rice varieties through backcross breeding using MABC (Xu et al. 2006).

Many DNA markers (gene-based or simply linked to the trait) have been developed, mainly for qualitative traits. Genome sequences for most major crops and great progress in NGS technologies have accelerated the systematic development of useful markers on a large scale. However, DNA markers have limited applications for selecting polygene traits of low heritability.

1.3.4 Genomic Selection

With rapid progress in NGS technology, we are able to obtain sequence information for large numbers of germplasm lines at a reasonable cost whenever needed. NGS technology revolutionized genomics and related studies. The genome-wide association study (GWAS), a statistical examination of the association of an array of single-nucleotide polymorphisms with a trait of interest, opened a new horizon for dissecting polygenic traits into genomic information. Until GWAS became available, detailed QTL studies were the only way to interpret quantitative traits and develop appropriate markers. Similar to GWAS, where all of the major- and minor-effect QTLs can be identified, genomic selection uses high-density marker sets for simultaneous selection of trait-enhancing loci across the genome (Heffner et al. 2009). Genomic-estimated breeding values (GEBVs) for the target traits can be estimated for each breeding line through genome selection, allowing breeders to select promising genotypes with higher accuracy. Instead of full-genome sequencing by NGS, genotyping-by-sequencing (GBS) is preferred for GWAS and genomic selection because of its relatively low cost and analytical simplicity (Elshire et al.

2011). Syngenta developed a drought-tolerant maize variety 'Syngenta-Artesian Corn Hybrids' using genomic selection.

1.3.5 Heritability and Genetic Gain After Selection

Genetic gain after selection of polygenic traits, which represent most agronomically important characteristics, such as yield, is greatly affected by heritability, the barometer of selection effect. To improve selection efficiency, populations or lines for selection should be cultivated under uniform/controlled environments and/or generation-advanced to increase heritability as much as possible. Even though genomic selection for polygenic traits is performed using GEBVs, genetic gain will be greatly influenced by heritability if the analysis used for GEBV calculation was based on phenotyping in the field. Therefore, GEBV calculations should be performed using the training populations grown in a uniform environment, where heritability estimates can be maximized by minimizing the environmental effects on phenotype.

1.4 Challenges and Perspectives

Breeders are facing global weather changes, increased food demand, limited natural resources such as water and energy, and new demand for health crops. Thus, breeding remains an essential task. Most modern elite varieties were developed by conventional breeding, which is still highly effective. However, conventional breeding products may not be able to meet current demands, so molecular breeding tools need to be actively utilized in current breeding programs. The methods and strategies discussed in previous sections are focused on creating new variation and selecting progeny with superior genotypes or phenotypes. Here, we will discuss the current challenges in breeding, focusing mainly on creating variation and selection methods by both conventional and molecular methods.

1.4.1 Creation of Variation

1.4.1.1 Recombination

Meiotic recombination is a basic mechanism for creating new variation by reshuffling homologous chromosome segments. Breeders are eager to bring desired alleles together into new combinations and to maximize reshuffling of alleles to create new variability. The frequency of meiotic recombination is determined by the number of chromosomes (via independent assortment) and the number crossover events along

a chromosome. Thus, variation can be created naturally by recombination events though the outcomes are quite variable and cannot be estimated.

Recently, IRRI celebrated their 100,000th crossing of rice, a distinguished achievement in hybridization breeding by a single institute. Given the number of rice crossings worldwide, we may be approaching the maximum variation possible through recombination. Although no direct evidence addresses this concern, it may explain why grain yield in rice has stagnated for decades. Similarly, the total number of crossings conducted per crop worldwide, including public and private institutes and universities, must be tremendous. Breeders may have already experienced most of the variation among frequently used germplasm, which can be induced by conventional breeding methods.

We must not overlook numerous crop germplasm resources, including wild relatives. Given that million of accessions exist, a tremendous amount of novel variation still remains to be exploited. Efforts are ongoing to use various germplasm accessions, such as land races or wild relatives, in which linkage drag would be a distinct barrier. With advanced DNA markers, MABC would increase the use of wild relatives or land races to enhance the types and range of genetic variation. Nonetheless, optimal genotype combinations might not be created without random recombination events or controlling meiotic recombination to modify the gametic allele compositions. Recently, the possibility of controlling meiotic recombination by increasing crossover incidence, altering crossover positions on chromosomes, and silencing crossover formation was reported (Wijnker and de Jong 2008; Osman et al. 2011). However, no variety or new phenotype variation has yet been developed this way. However, controlling meiotic recombination will be an important technology for creating novel variation that overcomes linkage drag and linkage blocks in the near future.

Epistasis, interactions among genes, has long been recognized as fundamentally important to understanding both the structure and function of genetic pathways and the evolutionary dynamics of complex genetic systems (Phillips 2009). Breeders make crosses or transgenic plants to introduce useful genes/alleles and to harmonize gene/allele combinations for variation and better performance in the progeny. However, epistasis is not well investigated as a genetic reservoir, even in intervarietal breeding. If recombination-controlling techniques are available, breeders will have more opportunity to produce desirable phenotypic variations and, thus, new epistasis interactions for yet more variation. Recent studies on meiosis and recombination may shed light on how to manipulate crossovers during meiosis (Osman et al. 2011; Crismani et al. 2013).

1.4.1.2 Mutation and Genome Editing

Mutation is an effective way to induce variability into plants. Along with cross-breeding, mutation breeding is a main method in conventional breeding. Many varieties that were developed by induced mutation have been released so

far. However, the occurrence of novel variations from this method has gradually decreased. The success of mutation breeding largely depends on a high mutation rate and technologies for detecting mutants. Even though various chemical mutagens and radiation types are available for mutation breeding, the mutation spectrum is limited for a few reasons. (1) Most induced mutants result in loss-of-function mutations, so gain-of-function mutants are rare. (2) Methods for screening mutants are limited. Physical appearance is the primary criterion for detecting mutants and only some biotic or abiotic stresses can be used for screening. Thus, without new large-scale screening methods, detecting mutant phenotypes will be limited. Recently, TILLING (Targeting Induced Local Lesions in Genomes), which can screen the mutated genes of known function and sequence in a high-throughput manner, became available as a new strategy. If breeders intend to knockout a specific gene or gene groups, targeted mutagenesis is a feasible approach that can be applied in plants via homologous recombination (Terada et al. 2002). However, this technique is being replaced by genome-editing techniques because of its low efficiency.(3) Finally, even without considering transposable elements, there may be mutable loci responsive to specific physical or chemical mutagens. In this case, other types of mutagens can be used to induce novel mutants. For example, ion beam mutation generated many novel mutants (Yu 2006).

Genome editing of a target area is expected to be a powerful tool to create desirable variation through insertion, replacement, or removal of genes from a genome using molecular scissors, which are artificially engineered nucleases. Three families of engineered nucleases are being used in plants: zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR/Cas system (Puchta and Fauser 2013).

1.4.1.3 Genetically-Modified Organisms (GMOs)

Transgenic breeding is appealing to breeders for creating totally new variation that cannot be obtained with conventional breeding. However, the issue of conventional versus transgenic breeding is still debated in both civilian and scientific communities. Debates about food/feed and environmental safety continue, despite scientific efforts toward providing an objective view on the issues (Nicolia et al. 2013). Some GM crops have been highly successful in assuring crop yield via protection against diseases, insect pests, and weeds by using alien genes. Increasing crop yield potential is the ultimate target for the future. For this, GM crops should incorporate genes to increase the efficiencies of nutrient and water uptake, nutrient use, photosynthesis, and translocation of photosynthates into storage organs. In addition, more effective and safer transgenic technologies should be developed to provide more new variants. Much as agrochemicals gained acceptance decades ago, the hope is that GM crops will be accepted in the near future because few alternative technologies exist for enhancing crop productivity.

1.4.2 Selection Methods

Breeders select improved genotypes based on phenotypes under specific or diverse environments, followed by regional performance tests before variety registration. During field surveys of early breeding generations, breeders observe and evaluate more than 100,000 lines, and genotype-by-environment interactions must be considered, so selecting the best individuals is difficult. Recently, DNA markers have enabled breeders to indirectly select desirable genotypes prior to field evaluations of traits. In most crops, however, only a small portion of genes in the genome have been associated with agronomic traits, so few markers, including linked markers, are available for MAS. Most genes important for agronomic traits remain unidentified.

In gene isolation studies, accurate phenotyping is critical because phenotypic values vary along environmental gradients in breeding fields. Therefore, stable, reproducible, and large-scale controlled environments that minimize environmental effects are crucial for determining genotype from phenotype. Moreover, phenotyping should be performed in a high-throughput system aided by remote-sensing and computer-based technologies. In reality, however, such facilities are difficult to establish. Currently, phenotyping is a limiting factor in isolating genes and subsequently developing DNA markers.

Genomic selection for quantitative traits is promising because even minor loci influencing a trait of interest can be identified and selected through whole-genome scanning. However, despite the feasibility of genomic selection, its effectiveness is limited for low-heritability traits (Nakaya and Isobe 2012). Therefore, genomic selection models should be developed using methods to eliminate environmental noise. Such markers may improve genetic gain by selection to the full extent of heritability. In addition, understanding gene-by-environment interactions would help in adopting genomic selection methods in diverse breeding fields. Recently, the epigenetic nature of agronomic traits is receiving much attention. However, whether epigenetic variations are heritable remains under study (Springer 2013).

When both genomic and epigenomic data on quantitative traits are thoroughly understood, breeders may overcome the limitation in selection gain caused by heritability.

1.5 Conclusion

Transgenic-based technologies to create novel variation, such as meiotic manipulation, targeted mutation, and genome editing, have the potential to replace traditional methods that mainly depended on the hybridization of germplasm and random mutation. Despite controversies about GM crops, transgenic technologies promise to be the basic tools for plant breeding in the future. Because GM technology has been successful for traits controlled by major-effect genes, it should be developed further so that multiple genes or poly genes can be harmoniously incorporated into target plants. Genome editing seems promising in this regard. In association with an understanding of epigenetic control of trait expression, the technology will initiate

a new era in designing useful polygenic variation. Creation of harmonious and desirable gene assemblies and gene networks will provide breeders with more opportunities to select better genotypes in their breeding fields.

Genomic selection is a state-of-the-art technology that enables breeders to select quantitative traits based on genotypes. To improve the accuracy of genome selection, phenotyping technologies should be systematized and automated in controlled environments, a field known as 'phenomics'. In addition, understanding epigenomic control and gene-by-environment interactions of target traits should accompany improved selection efficiency. Nonetheless, phenotypic validation, which includes stability and adaptability tests across locations and years, will remain the most important step in developing new varieties.

During the past century, plant breeding objectives have gradually changed to address global food security, industrial needs, and human preferences. Recent trends in global warming and frequent unfavorable climatic conditions compel breeders to adjust their targets and speed the development of new varieties for sustainability. The global seed market has been growing rapidly, with an annual increase of more than 10 %. A worldwide patent system for plant variety was consolidated by The International Union for the Protection of New Varieties of Plants (UPOV), stimulating private breeding companies to create competitive varieties. Future breeding programs should be more systematized and armed with new technological tools. Much as a car production line is automated, we anticipate that a series of technologies for breeding crop cultivars will be assembled and automated to produce designer varieties in the future (Fig. 1.2).

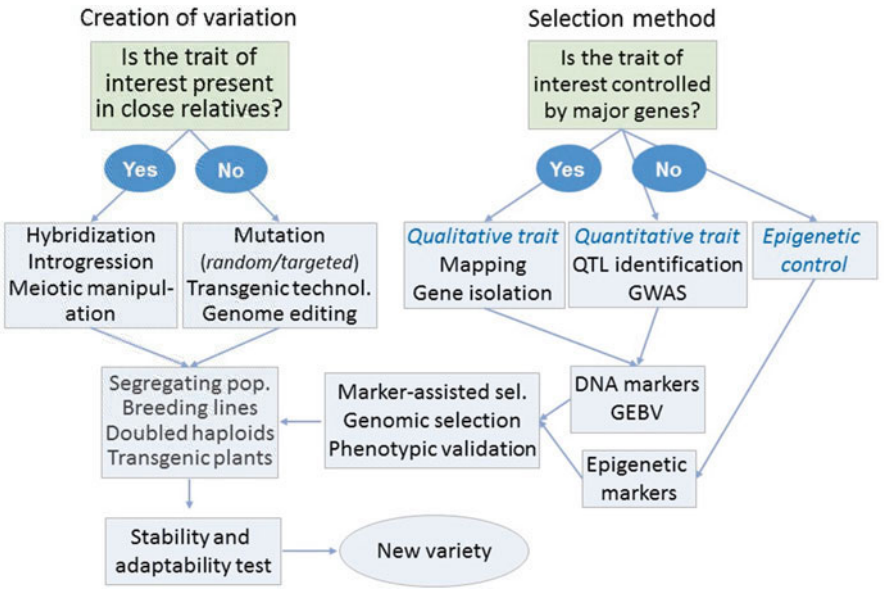

Fig. 1.2 Plant breeding in the twenty-first century (QTL, quantitative trait locus, GWAS, genome-wide association study; GEBV, genomic estimated breeding value)

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Chapter 2
Methods for Developing Molecular Markers

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Abstract Molecular markers are essential for breeding major crops today and many molecular marker techniques have been developed. DNA markers are now the most commonly used. This chapter describes the principles of DNA marker techniques and methods to map major genes. DNA markers can be classified into two categories: (1) DNA hybridization-based techniques, including restriction fragment polymorphism and DNA chips, and (2) polymerase chain reaction techniques, including simple sequence repeats, random amplified polymorphic DNA, amplified fragment length polymorphism, and single nucleotide polymorphism. To develop trait-linked markers, segregating populations for the target traits and reliable phenotyping methods are indispensable. With these tools, two approaches can be used to develop trait-linked markers: (1) when there is no biological information for the trait, and (2) when biological information is available. Finally, we describe several case studies for trait-linked marker development.

2.1 Definition of Technology and Related Terminology

When a specific phenotype, such as disease resistance or crop quality in plants, is difficult to determine, a different method must be used to investigate the trait. Genetic markers are a viable alternative. Because they are located close to the target gene and are inherited with it, selecting plants with useful traits using genetic markers is relatively easy. Genetic markers can be classified into morphological markers, including plant shape and/or color; protein markers, such as isozymes; and DNA markers based on sequence differences.

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Morphological markers were used first for genetic analysis by geneticists like Gregor Mendel and Thomas Hunt Morgan. However, their potential numbers are low, so few examples of their practical use exist. Protein markers such as isozymes, which were developed later, can distinguish individual plants. Thanks to this method, many samples could be analyzed with low cost. However, the relatively small number of isozyme variants limits their utility. DNA-based restriction fragment length polymorphism (RFLP) markers were developed and used in the 1980s, and in the following decade, polymerase chain reaction (PCR) technology gave rise to various types of DNA markers. The strengths and weaknesses of each type of genetic marker can be seen in Table 2.1.

Typically, the term 'molecular markers' indicates technology that uses phenotype-determining or closely related genes to find similarities or differences among individual plants, cultivars, or breeding lines. By analyzing molecular markers, individual plants with useful phenotypes can be selected at the seedling stage. Molecular markers for plant breeding are based on genetic differences among individuals in alleles at a certain locus. A molecular linkage map can be constructed by investigating the marker genotypes of each individual plant and calculating the genetic distances between marker pairs based on the recombinant frequency.

Table 2.1 Comparison of different types of genetic markers

Type	Benefit	Drawback	Example
Morphological markers	Easy to assay	Highly dependent on environmental factors	Color, shape, etc.
	Low cost	Difficult to analyze for quantitative traits	
		Difficult to determine heterozygosity	
		Limited availability	
Protein markers	Low cost	Assay samples must be in good condition	Isozymes
	Co-dominant	Limited availability	
	Less dependent on environmental factors	Unstable materials (protein)	
DNA markers based on hybridization	Do not require sequence information of the target	Costly and time consuming	RFLP
	Co-dominant	Use isotopes	
	Unaffected by environmental factors	Require large quantities of high molecular weight DNA	
		Difficult to automate	
DNA markers based on PCR	Require low quantities of DNA	Require expensive equipment	SSR
	Quick and easy to assay	Require sequence information	AFLP
	High accuracy		RAPD
	Unaffected by environmental factors		SNP

2.1.1 Types of Molecular Markers

2.1.1.1 Protein Markers

Proteins are the products of gene expression. Different alleles encode different amino acid sequences, giving proteins different sizes or biochemical characteristics that can be observed by electrophoresis and thus used as molecular markers. Isozymes are an example. Isozymes are useful molecular markers because they can be distinguished from each other based on differences in charge or size, despite having the same enzymatic activity. Although proteins cannot usually be seen by the naked eye, isozymes can be easily detected by separating via gel electrophoresis then adding the substrate of the enzyme. The isozyme produces color from the substrate, producing a band on the gel. Isozyme markers have a few drawbacks that greatly reduce their utility. In addition to having a very limited number of possible markers (only a few dozen have been developed), they are not distributed evenly on the chromosome, and often the enzyme activity depends on the plant's age or tissue type. Even so, isozyme analysis is very cheap and simple and was used for studies of maize, wheat, and barley decades before DNA markers were developed.

2.1.1.2 DNA Markers

DNA marker techniques use sequence differences among species or individuals within a species. Genetic differences among individuals in a group are usually due to abnormal pairing of sister chromosomes or recombination that rearranges the chromosomes, for example, insertions, deletions, inversions, translocation events, or reduplication. Chromosomal rearrangements can vary in size, from just a few base pairs to millions. DNA mutations in the form of base pair substitutions also occur. To develop genetic markers using DNA variants, DNA hybridization or PCR techniques are often used. In DNA hybridization, a short DNA fragment that is homologous to the target DNA is used as a probe. The probe is tagged with a radioisotope and hybridized with the DNA being analyzed. DNA variations can be detected based on the target–probe hybridization or the size of the hybridized DNA fragment. RFLP is an example. PCR techniques require only a small amount of DNA and are relatively simple and inexpensive. They include using minisatellites or microsatellites, sequence-specific primers such as sequence tagged sites (STSs) or expressed sequence tags (ESTs), and random primers such as random amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP) to amplify the DNA fragment and analyze its variants.

Molecular Markers Using Hybridization Methods: RFLP The classic example of molecular markers using DNA hybridization, RLFPs are a first-generation technique and the basis of many DNA marker methods that are used today. To better understand RFLPs, restriction enzymes and Southern blotting must be clear (Fig. 2.1).

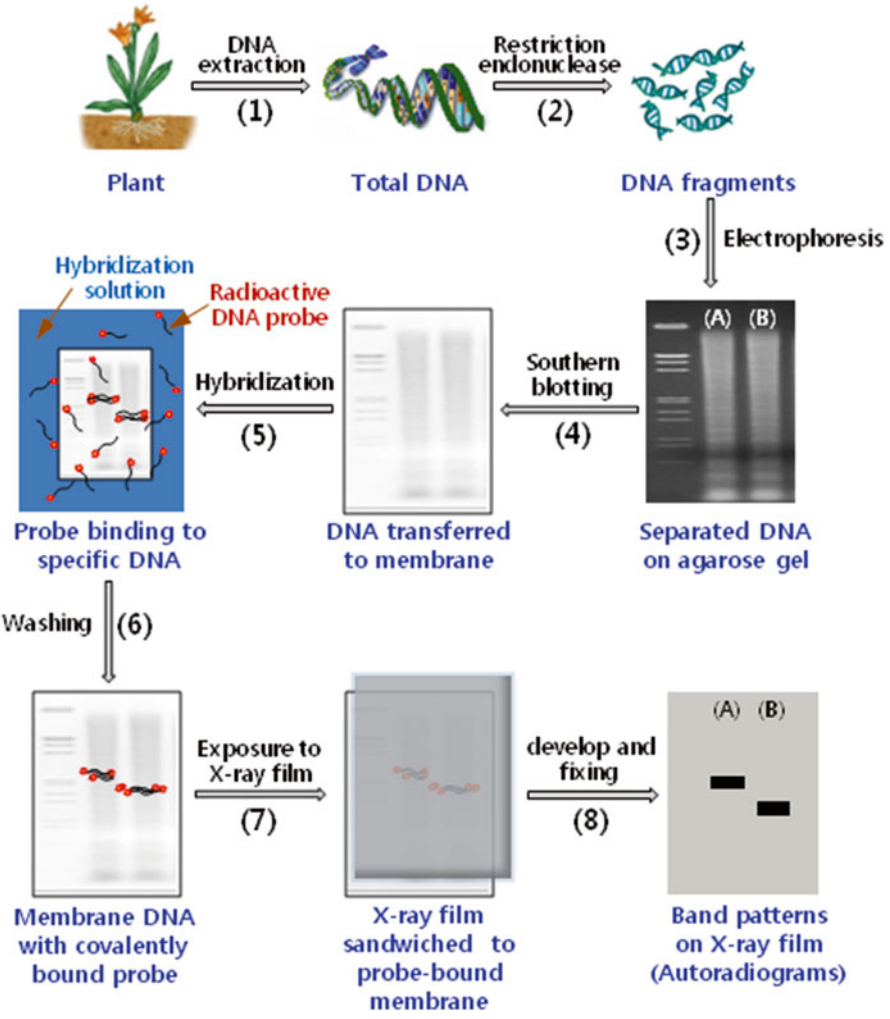

Fig. 2.1 Restriction fragment length polymorphism (RFLP) procedure. (*1*) Extract DNA from individuals A and B. (*2*) Use restriction enzymes to cut DNA. (*3*) Electrophorese DNA fragments on agarose gel to separate them by size. (*4*) Transfer the DNA in the gel to a nylon membrane by Southern blot. (*5*) Use radioactively labeled DNA fragments as probes to hybridize to the DNA. (*6*) Remove non-specifically bound or unbound probes by washing the nylon membrane. (*7*) Expose the washed membrane to X-ray film. (*8*) Develop the X-ray film to observe DNA polymorphisms

Restriction Endonucleases Restriction endonucleases (or restriction enzymes) are enzymes that recognize a specific DNA sequence (restriction sequence) and cut the DNA there. The recognized sequence can be four, six, or eight base pairs in length, depending on the enzyme. If a given DNA sequence has an equal numbers of A, T, G, and C, a restriction enzyme that recognizes six base pairs will cut every 4,096(4^6) positions on average, and the DNA of an organism with a genome size of 10^9 bp