Chapter 8
Molecular Breeding Strategies for Genetic Improvement in Rice (*Oryza sativa* L.)

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Abstract The current progress in crop research has provided a useful benchmark to evaluate crop-breeding improvement using genomics and molecular breeding techniques. The generation of huge amounts of molecular-genetic data has provided several ways to utilize the available genetic resources and to find solutions to the demanding goals of plant breeding. Rice being a staple food is consumed as an essential part of the dietary requirement by most of the developing countries. With the increase in population growth, traditional breeding methods cannot find a viable solution for sustainable crop production and food security. Since genetics and breeding are closely associated, combining these two has resulted in remarkable progress in rice-breeding programs. The presence of genetic diversity within cultivated crops and their wild relatives provides a platform for gene discovery of the agronomical important traits yet to be sufficiently discovered and utilized. This progress of developing new rice varieties with specific agronomic characters was made by using marker-assisted selection that opened new avenues for basic plant research. Combining conventional methods with molecular genetics will help in understanding the inheritance pattern of targeted traits in plant breeding and thus will lead to crop improvement in the future. This in turn can open new ways of improving the efficiency of breeding programs. Next-generation sequencing is the largest advancement and a boon for gene identification and variations in the genome. Recent techniques like CRISPR/Cas9 system are creating a major revolution in genome editing by adding or removing the genetic material at particular locations in the genome. Hence, molecular techniques are influencing the breeding process from selection to introgression of known genes/traits and thus sustaining the world's food productivity.

Keywords CRISPR · Crop improvement · Introgression · Microarrays · QTLs

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8.1 Introduction

Rice (*Oryza sativa* L.) is the staple food in many developing countries of the world, especially in Asia. It is a model cereal crop due to its small genome size, complete genome sequencing and high transformation frequency (Li et al. 2018a, b). Increase in global population up to 9 billion by 2050, along with water scarcity, diminishing arable land, adverse impacts of climate change, biotic and abiotic stresses present great challenges for rice breeders and agriculturists (Collard et al. 2008; Phillips et al. 2017). The crop yield per unit area needs to be increased by 50% by 2030 (Cheng and Hu 2008) to assure global food security. Hence, breeders are under increasing pressure to use new breeding strategies and enhance food production.

However, significant progress in recent years has been made by plant breeders to cope up with food shortages by combining breeding and molecular approaches. DNA-based markers have been developed for the construction of genetic maps and the accessibility to complete genome sequences have eased the work of breeders to introduce new crop varieties in a short time (Ashkani et al. 2015).

With the completion of whole-genome sequencing in rice, and low genotyping costs, various functional genomics platforms that include collection of germplasm resources, generation of mutant libraries, specific gene markers, QTLs, full-length cDNA libraries, gene expression microarrays and RNA-sequencing techniques for expression analysis have been developed in rice (Li et al. 2018a, b; Sun et al. 2015, 2018). Both genetics and genomics have contributed to crop breeding strategies for quality and quantity improvement (Fig. 8.1). Nowadays, nutritionally-rich rice varieties with favorable traits are available for human consumption (Gande et al. 2014; Hiwasa-Tanase and Ezura 2016).

8.2 Genetic Diversity

Large number of rice varieties are released every year to meet the increasing demands of enhanced productivity. All rice varieties have a different genetic composition, which is highly influenced by environmental conditions to which the plants are adapted. This variation in genetic diversity helps the plant to survive in nature. The advent of PCR (polymerase chain reaction) based molecular markers has increased the potential of discovering and tagging new genes in plants with diverse genetic makeups.

Wunna et al. (2016) observed high genetic diversity in landraces and rice varieties from upland and lowland ecosystems of Myanmar and, based on the extent of variations, the rice accessions were divided into two cluster groups, I and II, related to *indica* and *japonica* groups. Reig-Valiente et al. (2016) elucidated the genetic relationship in rice varieties consisting of modern elite and old cultivars and traditional landraces, cultivated in temperate regions. Whole genome sequencing and SNP (single nucleotide polymorphism) results revealed a strong substructure in

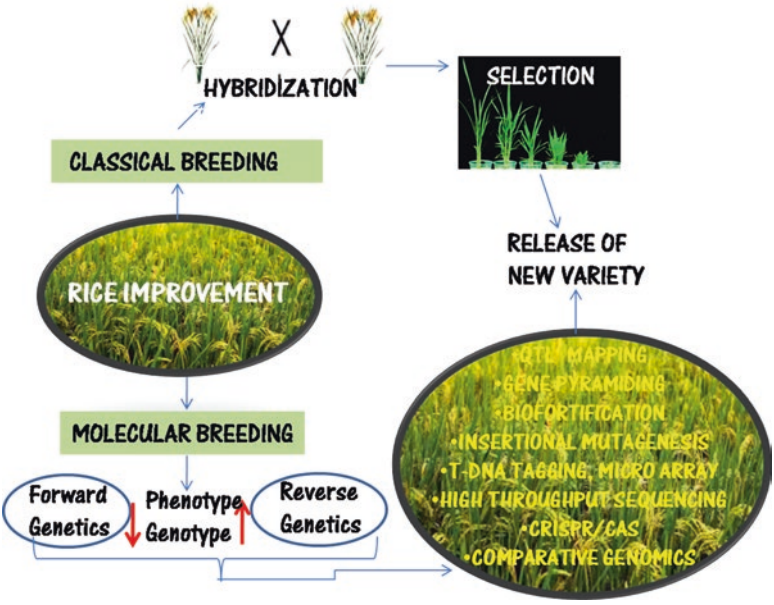

Fig. 8.1 Classical and molecular breeding approaches in rice: Continued improvement in rice involves the integration of different breeding approaches. Classical approaches involve hybridization followed by selection, while molecular approaches exploit rice genetics powered by functional genomics to meet the breeding challenges and safeguard food production

temperate rice populations which was based on grain type and the origin of the cultivars. A dendrogram also supported the population structure results. Aljumaili et al. (2018) studied the genetic diversity among aromatic rice accessions collected from Peninsular Malaysia, Sabah and Sarawak using SSR (simple sequence repeat) markers to quantify their genetic divergence. The presence of high genetic diversity could help in recognition of those accessions that can be used for introgression into the existing rice-breeding programs.

Indian rice varieties harbor huge amounts of genetic diversity but the trait-based improvement programs in recent decades have forced the breeders to rely on a few parents for crossing. As a result, there was a huge loss of gene diversity. Singh et al. (2016) studied the genetic diversity in 729 Indian varieties using HvSSR markers and divided them into two populations on the basis of cluster analysis. Anupam et al. (2017) genotyped germplasm of Tripura's local landraces, for genetic diversity and QTLs related to drought and blast resistance genes. However, a low level of genetic diversity was observed in contrast to high levels of genetic diversity among rice varieties in northeast India.

Genetic diversity is necessary for the survival of plant species under extreme climatic conditions or natural disasters. It acts as a natural defense system as it includes all the beneficial alleles for the plant species to thrive. With changing environmental conditions, only the best alleles are able to cope and adapt to new environments thereby playing an important role in evolution.

8.3 QTL Mapping

Molecular breeding in rice has played an important role in improving breeding efficiency. Many agronomic traits in rice are controlled by minor-effect genetic loci called quantitative traits, controlled by quantitative trait loci (QTLs) (McCouch and Doerge 1995). Hu et al. (2008) established a candidate gene strategy for the isolation of QTLs against bacterial blight and fungal blast. They observed that by combining different approaches like integrated linkage maps, expression profile and functional complementation analysis, a single minor QTL can be used in rice improvement. Zhou et al. (2014) studied in rice the inheritance of resistance to white tip disease in a segregating population derived from across between highly-resistant and susceptible cultivars. Six QTLs were detected after plotting on a genetic map which can be used for breeding resistant cultivars against white tip disease in rice.

Zeng et al. (2015) studied QTLs against sheath blight (SB) disease in a doubled haploid population from a cross between a *japonica* variety CJ06 and *indica* variety TN1, under 3 different environments. Based on SB resistance field data, a genetic map was constructed with 214 markers and it lead to the identification of 8 QTLs each against lesion height and disease rating, under 3 different environmental conditions. In addition, they also detected 12 QTLs for plant height, but observed that none of the plant height QTLs were co-located with the sheath blight QTL. Fiyaz et al. (2016) developed a population of 168 recombinant inbred lines from a highly resistant and a highly susceptible *indica* rice cultivar and mapped 3 QTLs against bakanae disease. Yao et al. (2016) identified QTLs associated with African rice gall mildew resistance in 3 independent bi-parental rice populations; 28 QTLs associated with pest incidence and pest severity were uncovered using composite interval mapping. A range of 1.3–34.1% of the phenotypic variance was observed with each of the individual QTLs.

High-throughput SNP genotyping has been used for various QTL mapping studies in rice (Chen et al. 2016). They performed high-resolution QTL mapping for grain appearance traits in *indica* rice. Duan et al. (2013) used high-throughput sequencing in the QTL mapping of a giant panicle rice accession, R1128, which has multiple major genes for good traits and detected 49 QTLs for 5 yield traits. For multiplexed samples, restriction-site associated DNA sequencing (RAD-seq) is a useful and cost-effective tool for genetic mapping that focuses only on short fragments of DNA adjacent to a particular restriction enzyme in the genome and allows efficient genotyping and high-density SNP discovery. Zhu et al. (2017) made a cross between *japonica* inbred Francis and *indica* restorer R998 and constructed a set of recombinant inbred lines so as to understand the genetic basis of rice yield traits in an elite restorer. A high density bin map was generated for QTL mapping of 6 yield-related traits.

Liu et al. (2006) analyzed QTLs against rice biomass yield, straw yield and grain yield in a population of 125 double-haploid lines from an inter-sub-specific cross of IR64/Azucena. A total of 12 QTLs were detected with additive main

effects, 27 involved in dysgenics interaction and 18 affected by the environmental conditions. Singh et al. (2017) mapped 38 and 46 QTLs on rice chromosomes 3 and 5 associated with tolerance to stagnant and irrigated conditions using a F7 mapping population. Gichuhi et al. (2016) identified 36 QTLs for yield-related traits from wild rice relative *Oryza longistaminata* for improving agronomic traits. Solis et al. (2018) identified QTLs associated with drought resistant traits by constructing a linkage map between two *japonica* cultivars with 213 markers. Composite interval mapping identified 6 QTLS associated with grain yield during drought conditions. Annotating candidate genes within QTLs suggested the role of genes and transcription factors that are involved in drought tolerance mechanism thus contributing to yield.

8.4 Assembling Multiple Desirable Genes by Pyramiding

Gene pyramiding has led to the development of genetic stocks and resulted in the improvement of plant breeding. Pyramiding involves stacking of multiple genes to develop durable resistance expression which further depends upon the number of genes to be transferred and the distance between the target genes and flanking markers (Joshi and Nayak 2010). Since traditional breeding is a time-consuming process, parent plants with desirable genes are crossed and the recombinants are selected from the progeny. As a result all the desirable genes are combined in a single breeding cycle, resulting in production of near recombinant lines (NILs) that contain homozygous alleles for the gene (s) of interest using fewer breeding cycles at lower cost. However, to track the presence or absence of a particular gene is slow. Although conventional breeding methods allow the transfer of desired genes between related species, genetic engineering balances and accelerates plant breeding programs by introducing genes from diverse sources. A large number of transgenic crops with durable resistance against biotic and abiotic stresses have been developed. Thus, it is possible to presume that genetic engineering is another useful method to pyramid novel genes into crop plants. However, efficient transformation and regeneration protocols need to be developed as a single gene transformation results in narrow spectrum disease resistance.

Huang et al. (1997) transferred 4 bacterial blight resistance genes (*Xa-4, xa-5, xa-13, Xa-21*) in rice and the pyramid lines showed a wider spectrum of resistance than lines with a single gene. They also developed PCR markers against 2 recessive genes (*xa-5, xa-13*) to screen a range of rice germplasm for the selection of parents in breeding programs. Pradhan et al. (2015) developed resistant cultivars in Jalmagna (an elite deepwater rice variety), against bacterial blight by transferring 3 resistance genes (*xa5 + xa13 + Xa21*) from the Swarna cultivar to develop a BB-susceptible cultivar, Jalmagna.

Fukuoka et al. (2015) developed near-isogenic experimental lines with 4 different QTL alleles and the lines were environmentally stable and resistant to blast disease. Similarly, Arunakumari et al. (2016) introgressed two bacterial blight

resistance genes (*Xa21*, *xa13*) and a major blast resistance (*Pi54*) gene into an Indian rice variety, MTU1010 through marker-assisted backcross breeding. Donor parents used were Samba Mahsuri (possessing *Xa21*, *xa13*) and NLR145 (possessing *Pi54*). The resultant lines had a high level of resistance against BB and blast, along with good yield, grain quality and plant type. Ji et al. (2016) developed 10 restorer lines by pyramiding genes resistant to blast, bacterial blight and brown plant hopper.

Gene pyramiding has played an important role in conferring strong and stable integration of alleles against resistance to various pests and pathogens. Yet, assembling large numbers of favorable alleles to provide stable agronomic performance is still time-consuming due to lack of molecular-marker information in certain plant systems. However, progress made in genome analysis by the use of high-throughput genotyping has contributed substantially to global food security.

8.5 Double Haploidy

Production of doubled haploids (DH) using anther or microspore culture under in vitro conditions is a successful and rapid approach to develop new rice cultivars, which otherwise requires at least 6–7 generations through conventional methods. The technique maintains the homozygosity that has wide uses in genetic studies, including gene and QTL mapping. In this method, the immature pollen grains or anthers (which are haploid) are cultivated in solid medium and induced to divide, to double their chromosome number so that the plants regenerated from them have two sets of chromosomes and hence double haploids.

Reiffers and Freire (1990) established a relation between the morphology of the panicle and the microspore stage. They observed that a cold-pretreatment of anthers at 4 °C for 8 days increased the regeneration frequency from 0 to 144.4%, while a few plants underwent spontaneous chromosome doubling. Chromium contamination in soil has become a severe threat to crop production and food safety. Qiu et al. (2011) detected 17 putative QTLs associated with Cr tolerance and Zn concentration using a rice DH population. Most of QTLs controlling Zn concentration had small genotypic variance and qSRZ4 related to Zn translocation showed growth condition-dependent expression. Fazaa et al. (2016) developed some DH lines through anther culture and evaluated them for yield and yield-related traits. Correlation analysis revealed grain yield to be positively correlated with panicle length. Grewal et al. (2011) produced double haploid lines through anther culture involving *indica* and *japonica* rice. They observed low anther culturability in the *indica* cultivars, as compared to *japonica* cultivars, and also variation regarding the Zn content in DH lines. A linkage map was constructed using SSR markers which revealed that the genes for anther culturability are partially dominant in *indica* cultivars and some DH lines had *indica* traits for high Zn content in polished rice.

Nguyen et al. (2016) created a DH population from the hybrid of *japonica* and *indica* rice, as earlier studies by Ikehashi and Araki (1986) indicated the presence of

a reproductive barrier between the two subspecies which was conditioned by the *S5* locus located on chromosome 6. Furthermore, it was observed that a neutral allele (*S5n*) can overcome this barrier because hybrids of either *indica* or *japonica* rice crossed to rice carrying *S5n* were fertile. Further genotyping of the *S5* locus, with allele-specific markers for *ORF3*, *ORF4* and *ORF5*, recorded a potential recombination hot spot in the *ORF3-ORF4* region. Haplotyping analysis revealed segregated distortion in the DH population, with a few lines having very low or very high *indica* alleles, with little effect of the *S5* allele. However, no effect of the $S5^n$ allele was observed on the agronomic traits studied.

Pauk et al. (2009) combined tissue-culture methods with conventional breeding to produce new rice varieties which are resilient than traditional ones. Risabell variety produced via anther culture was resistance to blast disease, high milling and cooking quality and long-grain type. Similarly for Janka variety, haploid cell cultures were developed and their vigorous regenerants were colchicine treated and the progeny has vigorous seedling growth, drought tolerance and good grain quality. However, for variety Ábel, the improvement was done through somatic tissue-culture regeneration followed by anther culture. Gueye and Ndir (2010) studied the response of anther culture of eight genotypes of *Oryza sativa* and *O. glaberrima* for callus induction and frequency of plant regeneration. They recorded more callus induction in *O. glaberrima* genotypes compared to *O. sativa* genotypes. Many albino plants were obtained while one *O. glaberrima* genotype (6202 Tog) produced green plants and thus it was concluded that the anther culture response is species and genotype dependent.

DH lines play an important role in inducing mutations thereby increasing selection efficiency. The technique is helpful to express popular recessive traits introduced through mutation or hybridization and hence enriching the germplasm. Even the double haploids govern major agronomic traits in cultivated rice that are influenced by QTLs. Also, double haploids overcome the problems of inbreeding by improving the selection efficiency in cross-pollinated species. The generation of DH lines plays an integral part in creating homozygosity as the purity of parental lines used in developing a hybrid/cultivar is reduced over the span of time due to gene drift, mutations, artificial breeding and exposure to various abiotic and biotic factors.

8.6 Increasing Nutritional Value with Biofortification

Rice is a major source of energy and micronutrients does not provide enough zinc to meet human nutritional requirements (Cakmak et al. 1999). This is due to the removal of large quantities of soil Zn by modern high-yielding varieties at each harvest, thereby lowering the residual concentration of soil Zn which further results in lower future grain Zn concentration (De Steur et al. 2014). Production of high-yielding rice varieties along with improved Zn concentration can be achieved when genetic and agronomic strategies are combined (Nakandalage et al. 2016). Screening

the germplasm, old landraces, traditional varieties and wild species using agronomic, breeding and genomic tools can increase Zn concentration in rice grain (Zaman et al. 2018). Further conventional plant breeding followed by marker-assisted selection has resulted in rice with enhanced level of micronutrients.

Several QTLs have been identified for high Zn in rice grain, which are used as a potential source for marker-assisted breeding (Chandel et al. 2011; Ishikawa et al. 2017). Identification of several candidate genes involved in Fe and Zn uptake and their accumulation in rice has led to their successful use in developing high Zn transgenic lines (Anuradha et al. 2012). Swamy et al. (2016) did a fine association of several gene specific markers with rice grain Zn. Similarly, several SSR markers and grain Zn trait associations identified in different populations and in a germplasm panel of rice can be further used in MAS (Brara et al. 2015; Khanin et al. 2016; Susanto 2008; Zhang et al. 2014).

Trijatmiko et al. (2016) reported Fe and Zn biofortification nutrition targets in rice under field conditions. The genes were introduced in IR64, an *indica* cultivar, and the trait was further bred into other popular rice cultivars deficient in Fe and Zn collected from South and Southeast Asia. An international initiative, The HarvestPlus program, aims to improve the micronutrient content of staple foods and reduce hunger. This program has increased Zn levels in brown and polished rice by 30 and 28 mg/kg, respectively (Johnson-Beebout et al. 2009). Trials are under way to identify and test certain sensitive biomarkers for Zn intake. However, experiments need to be carried out to evaluate the genotypes under Zn sufficient and deficient conditions at different stages of growth and development. Similarly, the interaction of environmental and genetic factors on Zn homeostasis needs to be established along with promoters and inhibitors of Zn bioavailability in rice grain (Nakandalage et al. 2016). Also, special attention should be given to the amount of antinutrients like phytate, as they significantly influence Zn bioavailability (Swamy et al. 2016).

Total Zn present in the aleurone layer, the outermost layer of the endosperm, is lost during processing, while that present in the inner endosperm (60–75%) is retained even after polishing (Hansen et al. 2009). This has been reported in large collections of rice germplasm at the International Rice Research Institute, Philippines (Boonchuay et al. 2013). The world's first Zn-enriched rice variety (BRRI dhan 62) was released in 2013 by the Bangladesh Rice Research Institute that contains 20–22 mg Zn per kg of brown rice.

Crops naturally lacking essential nutrients can use the transgenic plant breeding approaches to produce biofortified crops with desired nutrients and agronomic traits, and reduced antinurients (Bouis and Saltzman 2017). The expression of certain genes encoding enzymes involved in the synthesis or sequestration of IP6, have successfully reduced phytate concentrations in rice seed (White and Martin 2009). Golden Rice, rich in beta carotene, provides more than 50% increased vitamin A. However, the irony is that the Golden Rice has been available as a prototype since the early 2000s, but to date has been not introduced in any country due to the regulatory approval processes. Although transgenic varieties have great nutritional potential, their release to farmers will take several years, depending upon their approval through national biosafety and regulatory processes.

8.7 Induced Variations and TILLING

One of the efficient ways to study gene function is to create variations and then identify mutants that establish a link between genotype and phenotype. A potential approach to identify genes affecting trait variation is the use of induced mutants because they provide a clear understanding of molecular mechanisms involved in the plants (Sikora et al. 2011). Ethyl methyl sulfonate (EMS), is the most common mutagenic agent that induces chemical modification of nucleotides. The most common point mutations being GC to AT transitions. Once a gene structure is disrupted, the gene expression changes, resulting in changes in the phenotype which can be directly correlated to a gene responsible for it. However, knockout mutations are not always effective in functional analysis of the redundant genes because loss of function may not always lead to an obvious morphological variation, but also to the critical genes involved in plant growth and development (Wang et al. 2013). Induced mutants have been extensively used to identify gene(s) involved in various agronomically-important traits in rice. In India, six research institutes have collaboratively worked on an upland rice variety, Nagina 22, and identified mutants related to various important traits like plant growth and architecture, flowering, maturity, yield, grain number, shape and size, resistance to blast and bacterial leaf blight diseases and tolerance to drought and salinity (Mohapatra et al. 2014). The generated mutants, after registration, will be made available for various rice genetics and breeding programs.

Insertional mutagenesis, such as T-DNA insertions and transposon tagging, have been widely used in creating rice mutant libraries. Jeong et al. (2002) generated 13,450 T-DNA insertional lines using a new vector pGA2715 and suggested that the enhancer sequence present in the T-DNA improves the GUS-tagging efficiency. A reverse activation-tagging further identified the activation-tagged gene and enhancer effects.

Chen et al. (2003) characterized 1000 T-DNA tags in rice which were not spread randomly throughout the genome, but were inserted in gene rich areas. However, a few insertions, about 2.4%, were observed in repetitive regions. Good correlation was also observed in T-DNA insertions present in genic and intergenic regions with respect to size distribution. Krishnan et al. (2009) recorded the presence of insertion tags in 32,459 rice genes and also in 50% of predicted protein-coding genes with insertional mutagenesis. Chang et al. (2012) observed that out of a total of 372,346 mutant lines generated, 58,226 T-DNA or Tos17 flanking sequence tags have been isolated which have potential applications for more than 40 genes involved in stress responses, nutrient metabolism and plant architecture.

Characterization of the T-DNA insertion mutants resulted in identification of the biological functions of several genes. Wu et al. (2008) reported that there was no flowering in *rid1* mutant in rice while the *RID1* mutant was identified as a master switch that induces flowering. This was due to a regulatory gene *JMJ706* that resulted in H3K9 demethylation, a key step for development of rice floral tissues. Mutations in gene *JMJ706* carrying a T-DNA insertion resulted in altered floral

morphology and organ number due to increased di- and trimethylations (Sun and Zhou 2008). *ILA1*, a key factor for regulating the tissue formation at the rice leaf lamina, also showed abnormal mechanical tissue and cell wall composition in T-DNA mutant lines (Ning et al. 2011).

Targeting induced local lesions in genomes (TILLING) is a reverse genetic strategy developed to identify induced point mutations in a plant species, while the discovery and cataloguing of natural nucleotide variation present in populations is known as Ecotilling (Cooper et al. 2013). Till et al. (2007) developed two mutagenized rice populations on treatment with ethyl methane sulfonate (EMS), and sodium azide plus methyl-nitrosourea (Az-MNU) followed by further amplification of target regions of 0.7–1.5 kilobases using gene specific primers. They identified 27 nucleotide changes in the EMS-treated population and 30 in the Az-MNU population. Similarly, Cho et al. (2010) developed TILLING lines via the application of gamma-ray irradiation to rice seeds. The genetic diversity based on AFLP (amplified fragment length polymorphism) markers was assessed and changes in the coding regions of genes were observed with four loci exhibiting mis-sense mutations and two loci exhibiting silent mutations in the rice pseudomolecules.

8.8 Gene Discover by Next-Generation Sequencing

The dawn of next generation sequencing (NGS) has significantly revolutionized studies on rice functional genomics. With the release of genome sequences of *indica* and *japonica* rice, huge quantities of information and data are available and the variations in them can be identified by NGS efficiently and in a cost-effective manner. These variations are further used to identify the unique sequences for marker-assisted selection (MAS) in rice improvement programs (Spindel et al. 2016).

Rathinasabapathi et al. (2015) mapped the whole genome sequence of cultivar Swarna on the Nipponbare reference genome with high glycemic index (GI), and identified SNPs that could have a deleterious effect on protein functions. The changes in the position of SNPs in the granule bound starch synthase I gene and glucose-6-phosphate translocator gene contributed to a low GI. Similar variants were also observed in the genome of another *indica* rice variety collected from Columbia with low GI. Kharabian-Masouleh et al. (2011) observed SNPs and InDels in both coding and non-coding regions in candidate genes involved in starch synthesis. The *SSIIa* gene affected the starch quality and the amylopectin structure of starch present in the rice endosperm (Morell et al. 2003). The effect of this gene on cooking quality and starch content in the rice was studied by Umemoto and Aoki (2005) and further 31 SNPs and one InDel were detected in this gene.

NGS techniques could reveal the genetic basis of different phenotypes on the basis of DNA polymorphism under stress response conditions, even among closely-related cultivars. A contrasting response to drought and salinity stress in three cultivars of rice was studied by Jain et al. (2014). They observed that the distribution of SNPs and InDels was found to be uneven across and within the rice chromosomes.

These variations could be used as functional markers and to identify promising target genes for salinity and drought tolerance for molecular breeding programs.

NGS has enhanced the sensitivity of detecting mutations, thereby improving the screening efficiency by targeted gene amplification of pooled DNAs. Burkart-Waco et al. (2017) quantified and pooled DNA from a mutant rice population and amplified the target genes from these DNA pools. The amplicons were further combined to increase the probability of detecting mutations during sequencing. This approach can easily detect rare mutations. Ryohei et al. (2015) utilized the MutMap method, and its derivatives (MutMap+ and MutMap-Gap), to identify genes/QTLs of agronomic importance in rice (Abe et al. 2012; Fekih et al. 2013; Takagi et al. 2013).

8.9 Gene Introgression from Wild Relatives

Maintaining genetic integrity is essential for crops to sustain themselves in a changing environment. Alleles, introgressed for some beneficial traits, may result in genetic diversity. This changing diversity can satisfy the food needs of growing human population by delivering foods with high nutritional value and health benefits. All plants are domesticated from wild species, which are great reservoirs of genetic diversity due to stress and domestication traits, therefore the phylogenetic relationship between closely-related wild species is revealed by molecular analysis (Cheema et al. 2008; Dillon et al. 2007) that also gives an idea of their evolution under natural selection and their adaptation to the surrounding environment. Hence, conservation of wild relatives in the form of seed banks, or in situ and ex situ conservation is important for crop improvement (Brozynska et al. 2015).

Climate changes are harshly affecting food productivity, so it is of utmost importance to create crops which are genetically more diverse and resistant to biotic and abiotic stresses. Hence, there is an urgent need for the conservation of wild relatives from the available natural resources to ensure continuous food sustainability (Zhang et al. 2016). The consequences of 1.5 and 3.0 °C global temperature rise was studied for the coming years and then compared to the present climate. The results indicated an increase in taxa turnover and in the numbers of threatened taxa (Phillips et al. 2017). Thomas et al. (2017) studied the negative effect of climate on the distribution of genetic diversity in four wild relatives of rice and assessed a significant overlap between present and wild rice species. They observed that these species have a good opportunity to expand their distribution ranges in the near future to where rice is unlikely to be cultivated.

The distribution of genetic diversity within and among populations of the wild rice species *Oryza glumaepatula* in Costa Rica were observed by Fuchs et al. (2016). They evaluated that, how the incorporation of alleles from domesticated species may change the genetic makeup of wild species. A high level of genetic diversity was observed in *O. glumaepatula* populations in Costa Rica as compared to those present in South American populations, thus suggesting that the gene flow from cultivated *O. sativa* populations may have occurred in the recent past. This

could lead to the increase in likelihood of local extinction of pure *O. glumaepatula* populations, by the transfer of commercial traits from cultivated rice species.

Jin et al. (2018) determined crop-wild introgression from cultivated rice and its consequences in six *Oryza rufipogon* populations. Principal coordinates and cluster analyses indicated that the differentiation of wild rice populations that resulted in their altered genetic diversity is mainly associated with their spatial distances to cultivated rice fields. The level of overall genetic diversity detected with 34 SRRs and 34 In Dels recorded large wild-specific alleles in wild populations. Because crop-wild introgression can alter the genetic integrity of wild populations, appropriate measures need to be taken immediately for effective in situ conservation of pure wild relatives of crop cultivars.

8.10 Comparative Genomics

Comparative genomics is essential to study the minimal functions required by a plant for its normal growth, development and metabolism. It also explains the source of plant diversity and the molecular basis for its adaptation (Sasaki and Sederoff 2003). Rice has been used as a model crop for monocots to study its detailed structural and functional genomics due to the availability of its complete genome sequence, ESTs, transposons and production of transgenic plants (Shimamoto and Kyozuka 2002). However, previous studies revealed that the majority of rice genes are structurally and functionally homologous to major cereals, so the information obtained from rice genes can be easily utilized in studying their presence in other cereals (Hill and Li 2016; Wang et al. 2015). Comparative studies on the evolution of intergeneric regions in cereals has revealed that the large genome size in most crop plants is because of the presence of mobile elements rather than the functional genes.

Since *Arabidopsis* is a reference crop for dicots, the collinearity between the genomes of rice and *Arabidopsis* was studied by Liu et al. (2001) to compare and map rice BAC sequences with the *Arabidopsis* genome. Several regions were identified with preserved gene order but interrupted by non-collinear genes. Dodeweerd et al. (1999) investigated collinearity between rice and *Arabidopsis* by examining rice EST clones homologous to *Arabidopsis* genomic DNA sequences. A total of 24 homologous pairs, 5 with conserved order and a single inversion were observed. However, no conservation of gene order in rice and *Arabidopsis* across a 3-cM region in Chromosome 1 was identified by Devos et al. (1999).

Two genes *Hd1* and *Hd6* determining the flowering time were isolated by fine-scale, high-resolution mapping, corresponding to QTLs controlling the heading date of rice. It was observed that *Hd1* encodes a homolog of CONSTANS (CO) that functions in the photoperiodic control of flowering in *Arabidopsis* (Yano et al. 2000). Nelson et al. (2004) used sequence information from both the *indica* and *japonica* rice strains and identified 356 Cytochrome P450 genes and 99 related pseudogenes in the rice genome. When these rice genes were compared to P450

genes and pseudogenes in *Arabidopsis,* it was observed that many of the already-known plant P450 gene families existed before the divergence of monocot-dicot lineages took place. This study also highlighted the maintenance of certain lineage-specific families like Ranunculaceae and loss of lineage-specific families in *Arabidopsis* during the course of evolution.

Comparative genomics uses cross-genome comparisons of structure and function to estimate similarity of biological organization across species and genera (Wei et al. 2002). Sorrells et al. (2003) studied comparative sequence analysis of rice and wheat genomes and observed that wheat, a polyploid with a genome size 40 times larger than that of rice, has over 80% repeated DNA. Yan et al. (2003) studied microcollinearity in some regions between barley, wheat and rice. Analysis of the *Sh2/A1* orthologous region in rice, sorghum, maize and in some species of the Triticeae tribe revealed that this region was highly collinear with few differences (Bennetzen and Ramakrishna 2002; Li and Gill 2002)

Mayer et al. (2011) used the comparative genomics approach to unlock the genome of barley using a conserved synteny model with a model of grasses and assembled 21,766 barley genes in a putative linear order. It was observed that the barley genome exhibited a medley of structural similarity with hexaploid bread wheat. Thus the availability of the genomic resources for Triticeae plants after their genome sequencing have aided in the discovery of new genes using comparative genomics approach and in future this could further help in discovering alleles for adaptive traits to different agronomic environments (Mochida and Shinozaki 2013).

A centralized infrastructure, PLAZA (http://bioinformatics.psb.ugent.be/plaza/) is an online platform provides comprehensible and current research in the exploration of genome information (Proost et al. 2009). Here, all the data generated by different sequencing programs has been incorporated and combined and is further used for plant comparative genomics. This resource compiles structural and functional annotation of sequenced and published data and also maintains a large set of tools and softwares to study the function of genes and their evolution

8.11 Microarray and Gene Expression

High-throughput, genome-wide expression analysis in rice is facilitated by microarray technologies. The availability of complete genome sequences provides the necessary information required to design a microarray containing either all known or predicted gene models in the rice genome. A number of analytical tools have been developed to study gene relationships and functions from microarray data and the information obtained after analysis has been used in genetic dissection, drug discovery and disease diagnostics. Ma et al. (2005) analyzed the transcriptional activity of gene models and detected the expression of 41,754 known and predicted gene models. In addition, the expression patterns of best-matched homologous genes of rice and *Arabidopsis* indicated notable differences in the degree of conservation between

these two species. However, this lesser degree of conservation could be due to the diverged transposons and retrotransposons (Jiang et al. 2004).

Most of the genes, along with gibberellic acid (GA_3) and jasmonic acid (JA) play an important role in anther development and pollen fertility in rice. GA_3 controls the formation of pollen grains while JA signaling is required for pollen development and anther dehiscence. Wang et al. (2005) detected the expression level change of 2155 genes in anthers as compared to the seedlings using a cDNA microarray, with probes derived from meiotic anthers, mature anthers and treated suspension culture cells. A total of 314 genes responded to either GA_3 or JA treatment while 24 GA_3- and 82 JA-responsive genes were revealed. Furthermore, a significant difference was observed in the expression of GA_3 or JA treated genes at different developmental stages of anthers.

Microarray technologies facilitate high-throughput gene expression analysis but recent databases and softwares resulted in efficient expression analysis. There are different rice microarray platforms that can integrate microarray data for functional analysis and then can be effectively used in characterizing and differentiating the gene expression profiles from different rice tissues, organs, cell types, biotic and abiotic treatments, and miRNAs (De Abreu Neto and Frei 2016; Jung et al. 2015; Xue et al. 2009). The Rice Expression Profile Database (RiceXPro, http://ricexpro.dna.affrc.go.jp/), is a storehouse of different gene expression profiles of diverse organs and tissues at different developmental stages and environmental conditions (Sato et al. 2011). The Gene Chip rice genome array, designed by Affymetrix, contains 57,381 probe sets covering about 48,564 and 1260 transcripts from the *japonica* and *indica* cultivars, respectively (Cao et al. 2012), while the *Oryza sativa* Genome Oligo Set based on the draft *indica* and *japonica* sequences, was designed by the Beijing Genomics Institute and Yale University. Some other databases useful for expression pattern analysis of rice genes are OryzaExpress (Hamada et al. 2011), RicePLEX (Dash et al. 2012), Bio-Array Resource for Plant Biology (BAR) (Toufighi et al. 2005) and RiceArrayNet (Lee et al. 2009).

Another database Rice Oligonucleotide Array Database (ROAD, http://www.ricearray.org) is used for the exploration of gene expression based on rice microarray hybridizations. The database is user-friendly with a variety of tools that facilitate the study of gene expression profiles. ROAD supports analysis of genes expressed in different tissues, developmental stages and stress (both abiotic and biotic) conditions. Also, certain tools like Gene Ontology and KEGG (Kyoto Encyclopedia of Genes and Genomes) Orthology are fixed in the ROAD (Cao et al. 2012).

8.12 CRISPR for Targeted Genome Editing

Once sequencing of rice genome was completed, several tools became available for the functional characterization of genes. One of the most powerful and efficient tools which emerged recently for genome editing is CRISPR (clustered regularly interspaced short palindromic repeats). CRISPR has replaced the RNA interference

(RNAi) gene silencing technology for efficient and precise gene knock down by overcoming its limitations, like incomplete loss-of-function analysis and extensive off-target activities (Arora and Narula 2017). In rice, genes were modified for the traits related to biotic and abiotic stress, herbicidal resistance and yield, as well as genetic improvement of agricultural crops using CRISPR/Cas9 (Minkenberg et al. 2017; Xu et al. 2017) (Fig. 8.2). Efficient multiplex genome editing could be achieved by a synthetic gene that encodes for Cas9 protein with an intron containing polycistronic tRNA-gRNA in rice. Once a hybrid gene is formed, it could be expressed using one polymerase II promoter (Ding et al. 2018).

Rice seedlings are susceptible to low temperature, so a cold-tolerant transcription factor TIFY1b, involving genes was discovered in rice by Huang et al. (2017). They employed the CRISPR/Cas9 technique to edit this gene and also its homology gene in Nipponbare rice. High mutation rates due to insertion and deletion of one nucleotide were observed in transgenic lines. Thus CRISPR/Cas9 changed the DNA sequences at targeted sites and generated a variety of TIFY1 mutant lines in rice which were cold resistant.

Rice blast is one of the most destructive diseases affecting rice globally. Wang et al. (2016) reported the improvement of rice blast resistance by engineering a CRISPR/Cas9 and targeting the *OsERF922* gene for enhancing blast resistance. In addition, the number of blast lesions formed after pathogen infection was considerably decreased in all 6 mutant lines (identified from transgenic plants) both at the seedling and tillering stages. Also, no significant differences were observed between mutant lines and the wild-type plants with regard to the agronomic traits tested.

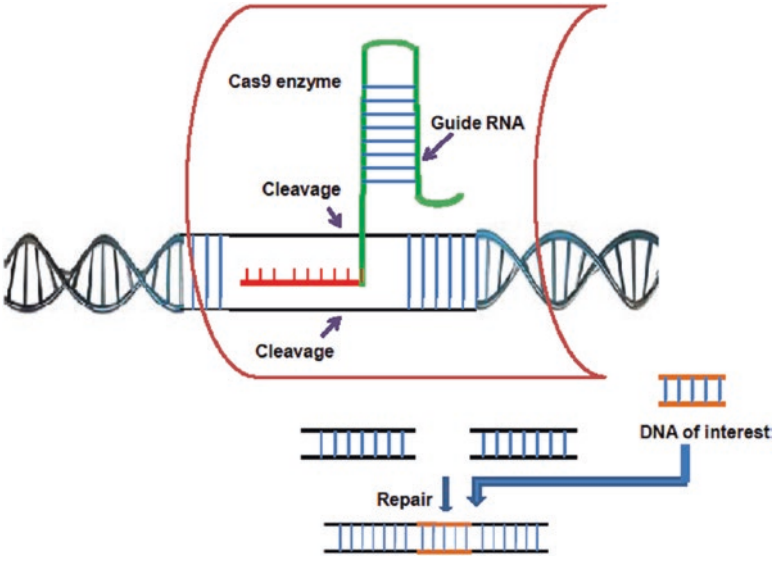

Fig. 8.2 CRISPR/Cas9, a RNA guided genome editing tool. CRISPR is a stretch of DNA while Cas9 is a molecular scissor that unwinds the DNA duplex and cleaves both the strands once the target sequence is recognized by the guide RNA

CRISPR/Cas system has been successful in rice protoplast cells with Cas9/sgRNA constructs targeting the promoter region of the bacterial blight susceptibility genes (Jiang et al. 2013). Genome editing generated the plants that were resistant to bacterial leaf blight, by down regulating the transcription of S-genes by the effector. Thus, the edited plants were resistant to certain bacterial strains because the effector was incapable of triggering the transcription of its target (Li et al. 2012). Similarly, multiple herbicide-resistant rice plants have been successfully achieved by CRISPR/Cas9-mediated in planta substitutions (Sun et al. 2016).

Cereals rich in amylose content offer potential health benefits. Previous studies using chemical mutagenesis have demonstrated that the fine structure and physical properties of starch is due to the starch branching enzyme (SBE) (Butardo et al. 2011). The targeted mutagenesis of rice *OsWaxy* gene by CRISPR/Cas9 resulted in high reduction in amylase content (14.6 to 2.6%) that resembles a natural glutinous rice variety (Ma et al. 2015). Sun et al. (2017) used CRISPR/Cas9 technology to generate targeted mutagenesis with In Dels in *SBEI* and *SBEIIb* in rice. Mutations were stably transmitted to the T1 generation. Wild type, sbeII mutants showed the presence of high amounts of long chains in debranched amylopectin in wild mutants that increased the amylase content by 25% in rice. The CRISPR/Cas9 technique has also been proved to be capable of editing and developing rice, photo-sensitive and thermo-sensitive male sterile lines to speed up breeding and exploit heterosis in rice (Li et al. 2016; Zhou et al. 2016).

Mutations resulting in complete knockouts and loss-of-function are very important to study gene functions, but their use becomes limited in many crop plants where gene expression is conferred due to point mutations. Multiple discrete point mutations in rice resulted from the introduction of the ALS gene using CRISPR/Cas9-mediated homologous recombination, which generated homozygous herbicide-resistant rice plants in one generation (Sun et al. 2016). Butt et al. (2017) applied CRISPR/Cas9 to generate targeted double-strand breaks and to deliver a RNA repair template for homology-directed repair in rice. For this, chimeric single-guide RNA molecules carrying sequences for target site specificity and repair template sequences flanked by regions of homology to the target were used. They concluded that this gene editing technology is very efficient in rice protoplasts to develop herbicide-resistant plants.

8.13 Conclusions and Prospects

This chapter emphasizes the recent advances and successful examples of molecular plant breeding that have led to significant improvement in rice. The adoption of molecular tools by breeders and researchers has helped them better understanding of the relationship between genotype and phenotype for complex traits, and the recent introduction of high-throughput genotyping platforms have increased the resources for plant breeding.

Similarly, the information extracted from genomics research has generated and added a wealth of information about gene structure and their functions, along with large numbers of molecular markers linked to QTLs. These generated resources will remain under exploited until breeding programs incorporate knowledge of pedigrees, phenotypes and marker genotypes during selection, and then combine them further with molecular approaches to discover new genes and their functions, which will further open innovative avenues for basic plant biology research.

In addition to the contributions made by breeders and researchers in adopting molecular approaches to meet certain plant breeding goals, the private sector should also participate by making investments to provide an appropriate training environment for agriculturists and scientists entering the molecular breeding workforce. This support can further bridge the gap between the latest techniques and the knowledge of plant molecular breeding research between the public and private sectors, and can help to meet the goals of sustainable increase in agricultural productivity.

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Appendices

Appendix I: Research Institutes Relevant to Rice Genetic Improvement

Country	Institution	Specialization and research activities	Contact information and website
Africa	Africa Rice Centre	Conserving rice genetic resources, rice breeding, rice processing	01 BP 4029, Abidjan 01, Côte d'Ivoire Tel: +225 22 48 09 10 Email: AfricaRice@cgiar.org
America	University of California	Breeding and genetics	G. S. Khush 39399 Blackhawk Place, Davis, CA 95616, USA Tel: (+1-530) 750-2440 Email: gurdev@khush.org
Arizona	The Arizona Genomics Institute	Facilitate the high throughput movement of genomic resources	1657 E Helen St,Tucson, AZ 85705, USA Phone: +1 520-626-9596
Brazil	Agronomic Institute of Paraná (IAPAR)	Improvement of agronomic traits	Lutécia Beatriz Canalli InstitutoAgronômico do Paraná – IAPAR Rodovia Celso Garcia Cid, km 375. Londrina-PR 86047-902, Brazil. Tel: (+55) 42 3219 9712 Email: lutecia@iapar.br

(continued)

Country	Institution	Specialization and research activities	Contact information and website
China	China National Rice Research Institute	Identification of genetic resources, investigation of new genes, functional genomic research	359 Tiyuchang Road, Hangzhou City, Zhejiang Province310006, P.R. China Tel: +86-571-63370212 Email: icoffice_cnrri@126.com
	Huazhong Agricultural University	Plant protection	Chao-Xi Luo Huazhong Agricultural University, College of Plant Science and Technology, Shizishan, Hongshan District, Wuhan City, Hubei Province, China 430070 Tel: (27)-87281242 Email: cxluo@mail.hzau.edu.cn
Germany	University of Freiburg	Coordinator of Golden Rice – Project	Peter Beyer Institute of Biology II (Cell Biology), Fahnenbergplatz, 79085 Freiburg im Breisgau, Germany Tel: +49 761 203 2529 Email: peter.beyer@biologie.uni-freiburg.de
India	Indian Institute of Rice Research	Genetic diversity, better rice varieties	V. Ravindra Babu Rajendranagar, Hyderabad, Telangana 500030 Email: director.iirr@icar.gov.in Tel: +91-40-24591218; Fax: +91-40-24591217
	National Research Centre on Plant Biotechnology	Genome sequencing and annotation of crop plants	N. K. Singh Indian Council of Agricultural Research, Pusa Road, New Delhi Tel: 011-25860186 Email: nksingh@nrcpb.org
Nigeria	National Cereals Research Institute	Yield enhancement and grain quality	DanbabaNahemiahBadeggi, Nigeria Tel: +234 806 931 4862
Philippines	The International Rice Research Institute	Plant breeder, Project leader for Green Super Rice	Jauhar Ali International Rice Research Institute, Los Baños, Laguna, Philippines Tel: +63 2 580 5600 ext 2541 Email:j.ali@irri.org
Taiwan	Institute of Molecular Biology	Rice transformation	Su-May Yu Institute of Molecular Biology, Academia Sinica, Nankang, Taipei 115, Taiwan Tel: 886-2-2788-2695 Email: sumay@imb.sinica.edu.tw

Appendix II: Rice Genetic Resources

Cultivation location	Cultivar	Important traits
Thailand	Dinalaga	Drought resistant
Africa	IRAT106	Drought resistant
Australia	Doongara	High amylase content
	Kyeema	Long grain and fragrant
Bangladesh	IR64-Sub1	Submerged
	BRRI dhan69	Saline, irrigated
	BRRI Dhan72	High Zn content
Brazil	Tre Smeses	Drought resistant
China	Yunlu 99	Drought resistant
	Huhan3	Drought resistant
Ghana	CRI-Emopa	–
	CRI Aunty Jane	–
India	Pusa Sugandh 2	Lodging tolerance, resistant to BB
	Ambemohar	Fragrant variety
	Pusa Sugandh 2	Lodging and shattering tolerance
	DRR-Dhan 45	Drought resistant
	Sampada	Low glycemic index
	CR Dhan10	Protein rich
Kenya	Komboka	–
Nepal	Sookha dhan4	Rainfed, drought
	Sookha dhan1	Drought
	Sookha dhan2	Drought
Nigeria	IAC47	Drought resistant
	Ofada	Highlyaromatic
Nigeria	UPIA1	Irrigated, rainfed, tolerance to toxicity
Philippines	NSIC Rc25	Upland
	NSIC Rc352	Irrigated, inbred
	NSIC Rc390	Saline
Thailand	Dinalaga	Drought resistant
Tanzania	Tai	Rainfed, irrigated
Uganda	Okile	–
Vietnam	08Fan10	Rainfed, lowland

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