Plant Genetics and Genomics: Crops and Models 5

Qifa Zhang
Rod A. Wing
Editors

Genetics and Genomics of Rice

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Foreword

Rice is the most important food crop and a staple food for more than half of the world's population. More than 90 % of the world's rice is grown and consumed in Asia, which is home to 60 % of the Earth's people. Rice accounts for 35–75 % of the calories consumed by more than three billion Asians. World rice production has increased three times from 231 million tons in 1961 to 718 million tons in 2011. This has mainly been achieved through the application of the principles of Mendelian genetics and conventional plant breeding coupled with improved production technologies. These advances in rice production have resulted in the Green Revolution. In 2000, the average per capita food grain availability was 20 % higher than in the 1960s. The resulting food security led to political stability, investment in education, infrastructure development, and industrialization in Asia. Despite these advances in rice production, 800 million people still go to bed hungry every day and most of them are poor rice consumers. It is estimated that we will need to produce 25 % more rice by 2030. Moreover, this increased demand will have to be met utilizing less land, water, labor, and chemicals. Furthermore, rice production and sustainability are continuously threatened by several biotic (diseases, insects) and abiotic (drought, submergence, salinity) stresses. These stresses are becoming increasingly important particularly in the context of global climate change. Thus, to overcome these constraints and ensure continued food security we need to develop genetically superior rice varieties with higher yield potential, possessing multiple resistance to biotic and abiotic stresses and with more palatable and nutritious grain quality. Thanks to the advances in rice genetics and genomics, we have new tools for developing rice varieties which will help us meet the challenge of feeding future rice consumers.

Rockefeller Foundation's International Program on Rice biotechnology (1985–2000) and the International Rice Genome Sequencing Project (IRGS, 2005) contributed much to the advances in rice molecular biology. Rice has become a model plant for genetic and genomic research in higher plants. I am delighted to see that two recognized authorities in this field have undertaken to prepare this authoritative review of the present status of rice genetics and genomics. The first six chapters review the further advances in genomics since publication of the rice genome sequence in 2005. Determining the functions of rice genes is now one of the major thrust areas in rice research. Four chapters are devoted to tools and resources for the functional analysis of rice

genes. Many useful genes have been identified for rice improvement. These have been catalogued in seven chapters. A thorough understanding of rice development and biological processes is crucial for future advances in rice research. Four chapters explore the present understanding of rice biology, while the last three chapters discuss their applications in rice improvement.

The write up of Perspective on synthesis and prospects is thought provoking and lays the road map for future advances in rice genetics and genomics.

This magnum opus should serve as a standard reference for rice researchers for many years to come. I would like to congratulate Drs. Qifa Zhang and Rod Wing for their labor of love in preparing this volume.

Davis, CA Gurdev S. Khush

Preface

Rice is the staple food for a large segment of the world population and global demand for rice production will continue to grow as we add more than two billion human inhabitants to the world population by 2050. Also increasing are the constraints for crop production posed by resource shortages and environmental degradation. As a response to these challenges, the international scientific community has made tremendous progress in rice functional and evolutionary genomics and biotechnology research over the last decade. This includes, but is not limited to, genomic resources such as a gold standard reference genome sequence, the generation of hundreds of thousands of mutant lines, collections of full length cDNAs, and databases for global expression profiles and natural variation. Hundreds of rice genes have now been cloned and molecularly characterized which have led to an enhanced understanding of agronomic traits and the underpinning of important biological processes. This book is devoted to a comprehensive coverage of the advances in such research.

The chapters are organized with the following considerations in mind: (1) rice is a model for genomic research of cereals for which we intended to present the features of the rice genome and the tools available and required for genomic studies; (2) rice is a crop that urgently needs genetic improvement for which we provide the current state of our molecular understanding of traits that are vital for varietal development; and (3) the model system of rice is different from Arabidopsis, and thus we must highlight and illustrate the advances in our understanding of the unique and important biological processes of this important cereal. We are very pleased that our goal has been achieved, thanks to the tireless efforts of the contributors.

This book is for the series on Plant Genetics and Genomics by Springer Publishing Co. Credit for initiating this effort goes to Richard Jorgensen, the Series Editor, and Amna Ahmed, Publishing Editor. This book enjoys the advantage that each chapter is presented by an authority on the subject with the latest developments. We sincerely thank all the authors for their dedicated efforts, and their time and talent in writing the chapters. We are particularly indebt to Gurdev Khush for his willingness to write the Foreword. We also thank Daniel Dominguez for his hard work in the communication and progress tracking.

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A Reference Rice Genome Sequence in the 10K Genome Era

1

Takeshi Itoh, Baltazar A. Antonio, Yoshihiro Kawahara, Tsuyoshi Tanaka, Hiroaki Sakai, Takashi Matsumoto, and Takuji Sasaki

1 Introduction

The completion of a high quality map-based genome sequence of *Oryza sativa* ssp. *japonica* cv. Nipponbare is one of the most important achievements in science with implications ranging from basic biology to the applied aspects of crop improvement in agriculture. The availability of the complete blueprint of all the genes of rice in the public domain provides an impetus that drives studies on structural, functional, and applied rice genomics. The rice genome sequence has also become a reference genome that is now being used in understanding the genome structure and function of major cereal crops including maize, wheat, barley, and sorghum. As rice has a very rich collection of germplasm resources, the Nipponbare genome sequence has proven to be an indispensable tool in identifying the genetic variability within and among the different species of the genus *Oryza*, an important step in looking for important agronomic traits in distantly related and wild rice species that can be incorporated in modern cultivars.

The completion of the genome sequence has also paved the way for more extensive projects on rice genomics. The *Oryza* Map Alignment Project has been initiated with the aim of using the Nipponbare genome sequence as a reference to characterize the wild species of rice and to create a genome-level experimental system for understanding the evolution, domestication, and genome organization of the genus *Oryza* [32]. The International Rice Functional Genomics Consortium (IRFGC) initiative that focused on sequence analysis of diverse rice cultivars of rice for the purpose of identifying SNPs based on 20 *japonica*/*indica* cultivars and landraces has elucidated the genotypic and phenotypic diversity of domesticated rice and allowed for identification of 160,000 SNPs [22]. More recently, the genome-wide association study (GWAS) has been widely adopted in rice as a strategy to characterize many common genetic variants across different accessions, to elucidate how these variants are associated with complex agronomic traits, and to reveal the heterogeneity of genetic architecture among diverse rice cultivars [10, 11, 36].

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2 Thousand-Genome Era

With the rapid advances in genome sequencing technology, the scenario has changed dramatically since the completion of the rice genome sequence in 2004. Genome researchers around the world are now focusing on sequencing not just a single representative of a species but rather hundreds and even thousands of closely related genomes or representatives of a single species [6, 7]. In plants, the 1,000 Plant Genomes Project is large-scale endeavor to obtain the transcriptome of representative species in the entire plant kingdom including algae, nonflowering plants, and angiosperms (http://www.onekp.com/). For the model dicot plant, the Arabidopsis genome research community has also embarked on a project that involved the whole genome sequencing of 1,001 Arabidopsis strains [31]. These highly ambitious genomics initiatives take advantage of next-generation sequencing technologies that could facilitate sequencing, resequencing, and assembling of entire genomes more accurately and efficiently, at a much lower cost and at a much greater depth.

With a large variation in the genus *Oryza* and the urgent need in agriculture to increase yield and improve the quality of crops, hundreds of cultivars and wild rice species have already been partially sequenced and analyzed by genome-wide association studies [10, 11, 33]. In addition, the genome sequences of more than 10,000 cultivars representing global rice genetic diversity are expected to be released in the near future. In particular, the "Rice 10,000 Genome Project" has been initiated as a joint collaborative effort of the International Rice Research Institute (IRRI), Chinese Academy of Agricultural Science (CAAS), and Beijing Genomics Institute (BGI) to characterize the global diversity of rice (http://bgiamericas.com/scientific-expertise/collaborative-projects/). This project would lead to the genome sequencing of modern rice varieties, traditional rice varieties, and wild relatives selected from the gene bank collections at IRRI and would guarantee more robust rice breeding programs.

The value of sequencing thousands of rice varieties could be enhanced if a high quality reference genome is available. Therefore, it is an urgent issue to provide a reference genome sequence with ultrahigh quality that can be used for many genomic studies such as large-scale SNP detections among cultivars. In this review, the major features of the reference rice genome sequence are described with emphasis on current initiatives to improve the sequence via concerted efforts to generate a unified genome sequence assembly. The annotation efforts of the Rice Annotation Project Database (RAP-DB) are described with new added features that will further enhance the genome sequence and provide more information on each predicted gene of rice.

3 Generating the Map-Based Genome Sequence

The initiative that led to the sequencing of the rice genome started in 1992 with the Rice Genome Research Program (RGP), a national project funded by the Ministry of Agriculture, Forestry and Fisheries of Japan with the aim of characterizing the rice plant, which is considered as the main pillar of Japanese agriculture. During the first phase of the program, it has successfully established a high-density linkage map of rice [9, 15], an extensive catalog of all expressed rice genes [34], and a yeast artificial chromosome (YAC)-based physical map covering the entire genome [16]. Even at the early stages of the project, these molecular tools were proven to be useful in understanding the genomic structure of rice and other cereal grasses, which were eventually found to share extensive similarity in their genetic make-up.

In 1998, the RGP embarked on the second phase of genome analysis, which was aimed at sequencing the entire rice genome. Even with a relatively small genome size estimated at 430 Mb, the task of sequencing the entire genome was undeniably enormous at a time when the high-throughput DNA sequencing technology was still at relatively early stages of development. The genome sequencing initiative in Japan served as a stimulus for the USA as well as other Asian and European countries to establish similar programs on rice genome analysis.

Eventually an international collaboration that has evolved into the International Rice Genome Sequencing Project (IRGSP) was organized with the aim of sharing resources and technology to accelerate the completion of sequencing the rice genome [26]. The IRGSP, formally established in 1998, pooled the resources of sequencing groups in ten nations to obtain a complete finished quality sequence of a single inbred cultivar, Nipponbare, and adopted a hierarchical clone-by-clone method using bacterial artificial chromosome (BAC) clones and P1 artificial chromosome (PAC) clones. This strategy employed a high-density genetic map, expressed sequence tags (ESTs), YAC-based physical map, and PAC/BAC-based physical map, BAC-end sequences, and draft sequences contributed by two commercial companies, namely, Monsanto [5] and Syngenta [8].

The map-based sequence of the Nipponbare genome consisted of 3,401 BAC/PAC clones which were sequenced to approximately tenfold sequence coverage, assembled, ordered, and finished to a sequence quality of less than one error per 10,000 bases [12]. A majority of physical gaps in the BAC/PAC tiling path were bridged using a variety of substrates including PCR fragments, 10 kb plasmids, and 40 kb fosmid clones. In total, the finished quality sequence covered 95 % of the 389 Mb genome including virtually all of the euchromatin regions and the two centromeres. A total of 37,544 non-transposable element-related protein coding genes were identified, 71 % of which had a putative homolog in Arabidopsis. Fourteen percent of the 37,544 predicted genes were found to appear in tandem duplications.

The publication on the rice genome sequence was based on the Build 3.0 assembly, which was also used as the template for manual curation of annotation in conjunction with the First Rice Annotation Project Meeting (RAP1) which was held in 2004. A year later, another update led to the construction of Build 4.0 genome assembly. Subsequently, the nucleotide sequences of seven new clones mapped on the euchromatin–telomere junctions were added, several clones in the centromere region of chromosome 5 were improved, and one gap on chromosome 11 was closed which led to the release of the Build 5.0 genome assembly at the end of 2008. The most recent version of the PAC/BAC-based physical map of rice that served as the template for genome sequencing is shown in Fig. 1.1. Although significant progress has been achieved since the initial publication of the entire genome, particularly in closing the gaps and characterizing the centromere and telomere-ends, some regions still remain to be completely sequenced.

4 Highly Accurate Genome Sequence

In order to further improve the quality of the map-based genome sequence, we resequenced the Nipponbare genome using the Illumina Genome Analyzer and obtained a total of 70 million 36-bp single-end reads and 60 million 51-bp single-end reads. These sequences were combined with more than 269 million 76-bp paired-end reads obtained independently by the Cold Spring Harbor Laboratory (W.R. McCombie, personal communication). After removing the low-quality sites and adapter sequences, the reads were mapped to the Nipponbare reference genome sequence by the Burrows–Wheeler Alignment (BWA) tool that could efficiently align short sequencing reads against a reference sequence [17]. The uniquely mapped reads could cover 90.6 % of the genome with an average depth of 43.6, which should be large enough to thoroughly validate the sequencing errors. We searched for sites covered by at least ten reads and found that 80 % or more were different from the reference genome sequence so that the total number of the nucleotide sites that could be used for further analysis were 321 Mb. As a result, a total of 3,447 sites were detected as SNP-type errors in the reference genome. In addition, a total of 1,439 small insertion/deletion-type errors were also found. Hence, a sequencing error rate of 1.5×10^{-5} per site was obtained. In addition to the Illumina reads, we also used 2,706,353 reads (1.0 Gb) generated by GS FLX Titanium. To detect large gaps, these reads were aligned to the reference genome by megablast [35]. After manual inspection of the results, merely five erroneous gaps remained and were corrected.

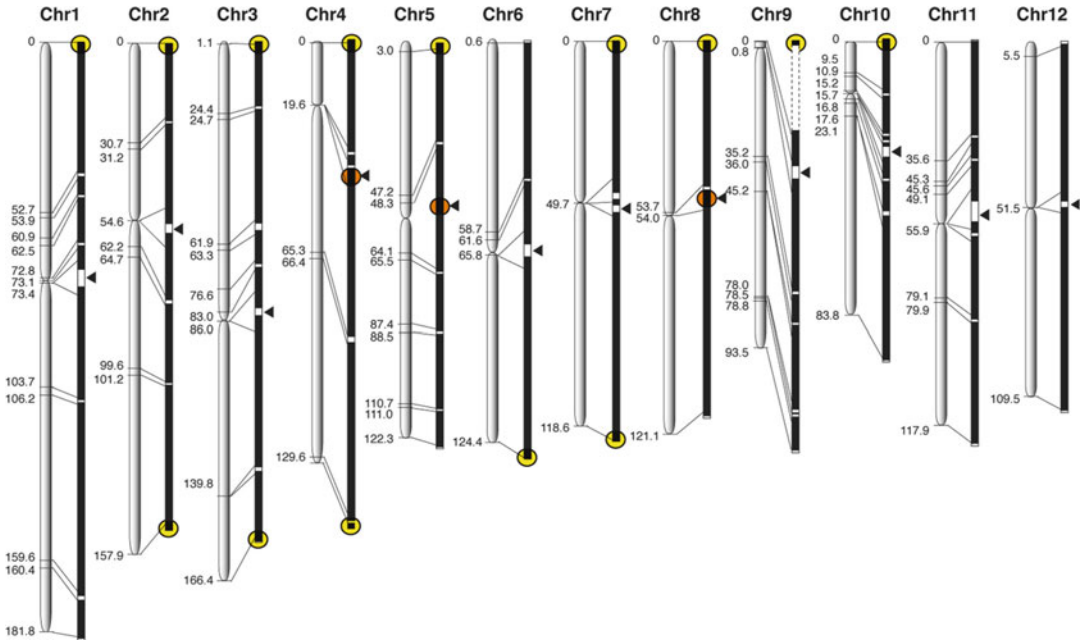

Fig. 1.1 Physical map of the 12 rice chromosomes. For each chromosome (Chr1–12), the genetic map is shown on the *left* and the physical map on the *right*. The position of markers flanking the gaps of the physical map which are shown in *white* is indicated on the genetic map. The nucleolar organizer on chromosome 9 is represented with a *dotted line*. Constrictions in the genetic maps and *arrowheads* to the right of physical maps represent the chromosomal positions of centromeres. The centromeres of chromosomes 4, 5, and 8 (*circled*) represent the first fully sequenced centromere reported in plants. The telomere-specific repetitive sequence unit, CCCTAAA, has been clarified in 14 telomeres. The genetic map is scaled to genetic distance in centimorgan (cM) and the physical map corresponds to the relative physical length

The IRGSP genome assembly was unified with the sequence assembly from the MSU Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/). Additionally, the assembly has been reconstructed using an optimal BAC tiling path that included a BAC-optimal map [37]. The unified high quality genome assembly, Os-Nipponbare-Reference-IRGSP-1.0 (IRGSP-1.0), can be downloaded at http://rapdb.dna.affrc.go.jp/ and http://rice.plantbiology.msu.edu/.

5 Annotation of the Os-Nipponbare-Reference-IRGSP-1.0

The annotation of the Nipponbare genome assembly has been available through the RAP-DB including automatically predicted and manually curated gene models [24, 29]. Re-annotation of the unified IRGSP-1.0 genome assembly was then conducted. The primary gene structures were determined by the cDNA-mapping method described previously [4, 29]. We used 207,343 major monocotyledon cDNA sequences registered in the international DNA databases (EMBL/DDBA/GenBank) including 81,129 cDNAs obtained from *Zea mays*, 39,676 from *Oryza sativa* (*japonica*), 30,270 from *Hordeum vulgare*, 26,321 from *Triticum aestivum*, and 11,789 from *O. sativa* (*indica*). Most of these sequences cover the full-length transcript [1, 13, 14, 18, 21, 23, 27, 28], so that it is expected that the complete exon–intron structure can be reconstructed on the genome. The protein sequences of plants in UniProt [30] and RefSeq [25] were also aligned to the genome so that the number of missing genes in the annotation data set could be

Table 1.1 Statistics of annotated loci in the Build 5 and IRGSP-1.0 assemblies

	Build 5.0	IRGSP-1.0
Protein coding loci	31,232	33,276
Nonprotein coding loci	1,515	2,191
Ab initio predictions[a]	2,034	2,405
Total	34,781	37,872

[a]The primary gene structures were determined by ab initio prediction methods and all of them are possibly protein coding

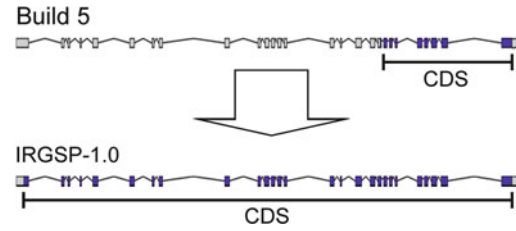

Fig. 1.2 Improved CDS regions by error-corrections in the IRGSP-1.0 assembly

maintained at a minimum. To further add genes without supporting cDNA or protein sequence information, ab initio gene predictions by GlimmerHMM, GeneMark.hmm, and GeneZilla were conducted [19, 20] and the prediction results were integrated by JIGSAW [2]. We compared the predicted genes with 6,700,357 ESTs of major cereals by BLASTN [3]. Predictions were employed in the final annotation data set for all annotations supported by the expression evidence of ESTs.

This comprehensive annotation resulted in identification and prediction of 37,872 loci (Table 1.1). In the previous assembly (Build 5), a total of 34,781 loci were predicted corresponding to 31,232 protein coding loci, 1,515 nonprotein coding loci, and 2,034 ab initio predictions. More than 3,000 novel loci were found in the current assembly because thousands of full-length cDNAs of maize and barley were newly added to the cDNA-mapping data set. In many cases, these novel loci were located in the opposite strand of other loci suggesting that they might be possible antisense transcripts. However it is also possible that these loci are transcribed specifically in non-rice species or are experimental artifacts that should be subjected to further validation.

The genome-wide error correction of the IRGSP-1.0 assembly improved the annotation. For example, a protein coding region of a locus (Os10g0477800) was truncated in the Build 5 assembly (Fig. 1.2), but it turned out that this coding-frame disruption was artificial. In fact, the coding region could be extended to the genuine amino-terminal in the IRGSP-1.0 assembly and the intact frame was recovered. Since the rice genome was deciphered by the Sanger method on the basis of a precise physical map, the sequence quality was thought to be high enough. Nonetheless, to cope with the continuous increase of sequence data from thousands of cultivars lined-up for sequencing, the reference genome assembly should be of exceptionally high quality to facilitate accurate comparative analysis among different cultivars.

6 Concluding Remarks

Rice biology is now in the midst of a genomics revolution with the cheap, fast, and ubiquitous sequencing of thousands of cultivars and lines that represent the *Oryza* germplasm resources. In the next few years or so, the public databases will be flooded with rice sequence data of almost all cultivars and lines grown around the world. A high quality rice genome sequence will always play a pivotal role in analysis of these genome sequence data. Available to researchers worldwide, the rice genome reference sequence provides an unprecedented biological resource to the scientific community that will serve as a basis for research and discovery of novel genes and, ultimately, introgression of these genes into cultivars grown in different cultivation conditions. The sequence already is having an impact on finding genes associated with many agronomic traits in rice. Other rice genome sequence projects focusing on various rice cultivars will enable detailed comparisons among cultivars, species, and wild relatives. It is therefore important that the Os-Nipponbare-Reference-IRGSP-1.0 genome assembly is of the highest quality. The next challenge will focus on how to

use all the information in actual breeding programs to facilitate the breeding of new varieties that are stronger, faster-growing, and higher-yielding than varieties currently available, to guarantee a stable food supply for mankind.

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The Wild Relative of Rice: Genomes and Genomics

2

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The wild species of the genus *Oryza* serve as a virtually untapped reservoir of genetic diversity that can be used to improve the world's most important food crop—rice. The genus is composed of two domesticated (*O. sativa* and *O. glaberrima*) and 22 wild species [68] and represents between 15 and 25 million years of evolutionary diversification.

In this chapter we will describe the current status of the genetic and genomic applications of the genus *Oryza* toward the penultimate goal of helping to solve the 9 billion people question—i.e., how can we grow enough food to feed more than 9 billion human inhabitants under 40 years [52]?

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1 The Genus *Oryza*: Broadening the Gene Pool of Rice—Exploitation of Diversity of the Wild Species Germplasm

The genus *Oryza* includes two cultivated ($2n = 24$, AA) and 22 wild species ($2n = 24$, 48) representing the AA, BB, CC, BBCC, CCDD, EE, FF, GG, KKLL, and HHJJ genome types (Table 2.1). Figures 2.1 and 2.2 show a phylogenetic tree of the genus (inferred from [3, 6, 25, 45]) and a photograph of 12 of these species at the same developmental stage, respectively. These wild *Oryza* species are, in fact, grass-like plants which are phenotypically inferior in agronomic traits—such as poor plant type, low grain yield, poor grain type, and are shattering in nature [1]. The wild species exhibit tremendous diversity in morphological traits, height, tillering, flowering, growth habit, panicle, leaf, culm, and seed characteristics (Fig. 2.2), and adaptation to different habitats and agronomic traits (Table 2.1).

2 Gene Transfer from Wild Species into Rice

The International Rice Research Institute's (IRRI) Rice Gene Bank and The National Institute of Genetics' *Oryza* base, combined, maintain more than 4,000 accessions of wild *Oryza* species and 1,500 accessions of cultivated

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Table 2.1 Chromosome number, genomic composition, and distribution of *Oryza* species, *Oryza*-related genera, and their useful traits

Species	2*n*	Genome	Number of accessions	Distribution	Useful traits
O. sativa complex					
O. sativa L.	24	AA	96,564	Worldwide	Cultigen, high yielding
O. glaberrima Steud.	24	A^gA^g	1,562	West Africa	Cultigen; tolerance to drought, acidity, iron toxicity, P-deficiency; resistance to BB, blast, RYMV, African gall midge, nematodes, weed competitiveness
O. nivara Sharma et Shastry	24	AA	1,260	Tropical and subtropical Asia	Resistance to grassy stunt virus, BB
O. rufipogon Griff.	24	AA	858	Tropical and subtropical Asia, tropical Australia	Resistance to BB, blast, BPH, tungro virus; moderately tolerant to Shb, tolerance to aluminum and soil acidity, increased elongation under deep water; source of CMS and yield-enhancing loci
O. breviligulata A. Chev. et Roehr. *O. barthii*	24	A^gA^g	218	Africa	Resistance to GLH, BB; drought avoidance; tolerance to heat and drought
O. longistaminata A. Chev et Roehr	24	A^lA^l	203	Africa	Resistance to BB, nematodes, stemborer, drought avoidance
O. meridionalis Ng	24	A^mA^m	56	Tropical Australia	Elongation ability; drought avoidance; tolerance to heat and drought
O. glumaepatula Steud.	24	$A^{gp}A^{gp}$	54	South and Central America	Elongation ability; source of CMS; tolerance to heat
O. officinalis complex					
O. punctata Kotschy ex Steud.	24, 48	BB, BBCC	71	Africa	Resistance to BPH, BB, zigzag leafhopper; tolerance to heat and drought
O. minuta J.S. Presl. ex C.B. Presl.	48	BBCC	63	Philippines and Papua New Guinea	Resistance to BB, blast, BPH, GLH
O. officinalis Wall ex Watt	24	CC	265	Tropical and subtropical Asia, tropical Australia	Resistance to thrips, BPH, GLH, WPH, BB, stem rot; tolerance to heat
O. rhizomatis Vaughan	24	CC	19	Sri Lanka	Drought avoidance, resistance to blast; tolerance to heat
O. eichingeri A. Peter	24	CC	30	South Asia and East Africa	Resistance to BPH, WBPH, GLH
O. latifolia Desv.	48	CCDD	40	South and Central America	Resistance to BPH, BB, high biomass production
O. alta Swallen	48	CCDD	6	South and Central America	Resistance to striped stemborer; high biomass production
O. grandiglumis (Doell) Prod.	48	CCDD	10	South and Central America	High biomass production
O. australiensis Domin.	24	EE	36	Tropical Australia	Resistance to BPH, BB, blast; drought avoidance; tolerance to heat and drought

(continued)

Table 2.1 (continued)

Species	2*n*	Genome	Number of accessions	Distribution	Useful traits
O. meyeriana complex					
O. granulata Nees et Arn. ex Watt	24	GG	24	South and South Asia	Shade tolerance, adaptation to aerobic soil
O. meyeriana (Zoll. et (Mor. ex Steud.) Baill.)	24	GG	11	Southeast Asia	Shade tolerance; adaptation to aerobic soil
O. ridleyi complex					
O. longiglumis Jansen	48	HHJJ	6	Irian Jaya, Indonesia, and Papua New Guinea	Resistance to blast, BB
O. ridleyi Hook. F.	48	HHJJ	15	South Asia	Resistance to blast, BB, tungro virus, stem borer, whorl maggot
Unclassified					
O. brachyantha A. Chev. et Roehr	24	FF	19	Africa	Resistance to BB, yellow stemborer, leaf folder, whorl maggot; tolerance to laterite soil
O. schlechteri Pilger	48	KKLL	1	Papua New Guinea	Stoloniferous
O. coarctata Tateoka	48	KKLL	1	Asian Coastal Area	Tolerance to salinity, stoloniferous
Leersia perrieri A. Camus	24	UNKNOWN	1	Africa	Shade tolerance, stoloniferous

BPH brown plant hopper, *GLH* green leaf hopper, *WBPH* whitebacked plant hopper, *BB* bacterial blight, *Shb* sheath blight, *CMS* cytoplasmic male sterility, *RYMV* rice yellow mottle virus

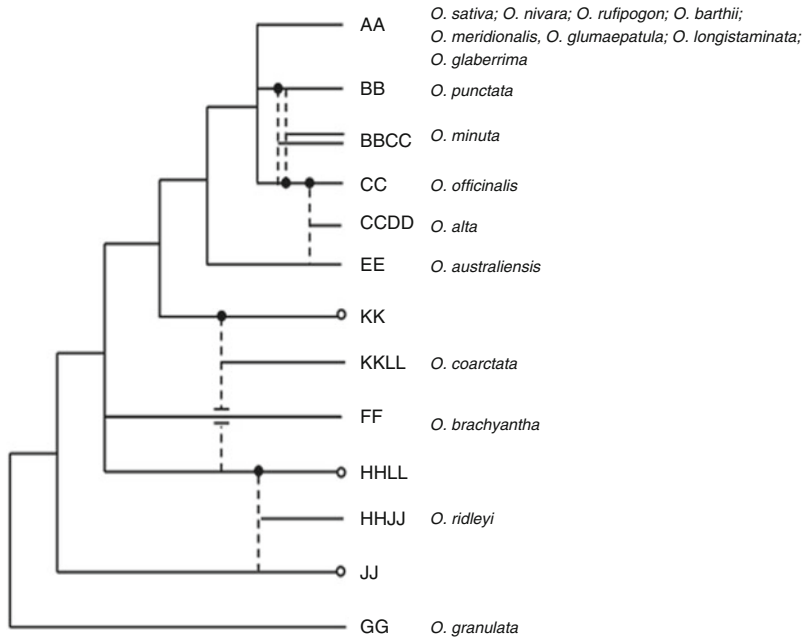

Fig. 2.1 *Oryza* phylogenetic tree. Evolutionary relationships of the *Oryza* genome were inferred from Ammiraju et al. [3, 6], Ge et al. [25], and Lu et al. [46]. *Dashed line* indicates origins of allotetraploids; *filled circle* indicates maternal parents; *open circle* indicates unidentified diploid species

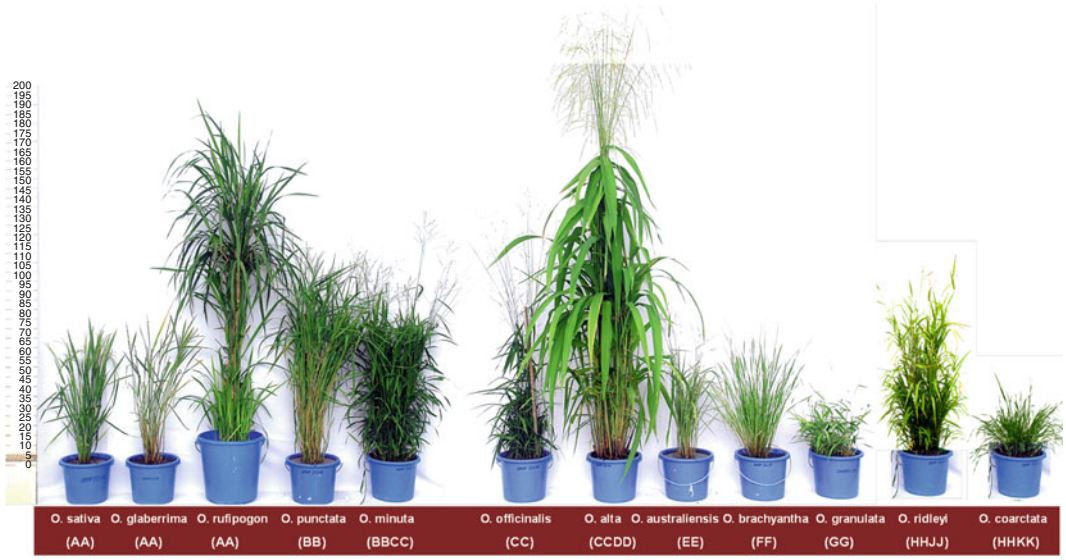

Fig. 2.2 The genus *Oryza*: 12 representative species

African rice (*O. glaberrima*). These wild species are reservoirs of many useful genes, particularly for resistance to major biotic and abiotic stresses (Table 2.1). However, these wild species are associated with several weedy traits, such as grain shattering, poor plant type, poor grain characteristics, and low seed yield. Besides, several incompatibility barriers limit the transfer of useful genes from wild species into cultivated species [14, 15]. The major consideration in alien gene transfer is to selectively transfer agronomically important genes from wild species while avoiding linkage drag. To achieve precise transfer of genes from wild species, strategies involving a combination of conventional plant breeding methods with tissue culture and molecular approaches have become important [16, 17]. Advances in tissue culture, molecular marker technology, genomics, and fluorescence in situ hybridization have opened new opportunities to tap alien genetic variability from distant *Oryza* genomes through interspecific hybridization.

3 Strategy for Alien Gene Transfer into Cultivated Rice

The strategy used to transfer genes from wild species into rice depends on the nature of the target trait(s), relatedness of the wild species, and incompatibility barriers. Several protocols are available to overcome such barriers [16]. Some of the steps involved in gene transfer include: (1) *Search for useful genetic variability for target traits*—this involves the screening of wild species to identify specific accession(s) that possess useful genetic variability. (2) *Production of hybrids and alien introgression lines* (*AIL*): interspecific hybrids are produced between elite breeding lines with the wild species carrying the desired traits. Such hybrids are produced through direct crosses between rice and AA genome wild species. However, embryo rescue is required to produce hybrids and backcross progenies (introgression lines) between rice and all the wild species of *Oryza* except AA genome species. (3) *Evaluation of introgression lines for transfer of target traits*: AILs generated through backcrossing are evaluated for the transfer of target traits. This involves extensive laboratory, greenhouse, and field testing. (4) *Molecular mapping of genes/QTLs*: molecular markers are developed to track the desired alien trait(s) for marker-assisted selection (MAS).

Following the above strategy, a number of genes have been transferred from wild species into rice (Table 2.2) and varieties have been released for commercial cultivation (Table 2.3). MAS has been practiced and some varieties have become available (Table 2.4).

Table 2.2 Introgression of genes from wild *Oryza* species into rice

	Donor *Oryza* species		
Trait	Wild species	Gene	Genome
Grassy stunt resistance	*O. nivara*	*GS*	AA
Bacterial blight resistance	*O. rufipogon*	*Xa23*	AA
	O. longistaminata	*Xa21*	AA
	O. nivara	*Xa38*	AA
	O. officinalis	*Xa29*(t)	CC
	O. minuta	*Xa27*	BBCC
	O. latifolia	Unknown	CCDD
	O. australiensis	Unknown	EE
	O. brachyantha	Unknown	FF
Blast resistance	*O. glaberrima*[a]	Unknown	AA
	O. rufipogon	Unknown	AA
	O. minuta	*Pi9*	BBCC
	O. australiensis	*Pi40*	EE
Brown planthopper resistance	*O. officinalis*	*bph11*, *bph12*	CC
	O. eichingeri	*Bph14*, *Bph15*	CC
	O. minuta	*Bph20*, *Bph21*	BBCC
	O. latifolia	Unknown	CCDD
	O. australiensis	*Bph10*, *Bph18*	EE
Whitebacked planthopper resistance	*O. officinalis*	*Wbph7*(t), *Wbph8*(t)	CC
	O. latifolia	Unknown	CCDD
Cytoplasmic male sterility	*O. sativa* f. *spontanea*	Unknown	AA
	O. perennis	Unknown	AA
	O. glumaepatula	Unknown	AA
	O. rufipogon	Unknown	AA
Tungro tolerance	*O. rufipogon*	Unknown	AA
Tolerance to iron toxicity	*O. rufipogon*	Unknown	AA
	O. glaberimma[a]	Unknown	AA
Heat and/or drought-related traits	*O. australiensis*[b]	Unknown	EE
	O. barthii[b]	Unknown	AA
	O. glaberimma[a]	QTLs	AA
	O. glumaepatula[b]	Unknown	AA
	O. meridionalis[b]	Unknown	AA
	O. officinalis[b]	Unknown	CC
	O. punctata[b]	Unknown	BB
	O. rhizomatis[b]	Unknown	CC
Tolerance to aluminum toxicity	*O. rufipogon*	QTL	AA
Tolerance to acidic conditions	*O. glaberrima*[a]	Unknown	AA
	O. rufipogon	Unknown	AA
Tolerance to P-deficiency	*O. rufipogon*	Unknown	AA
	O. glaberimma[a]	Unknown	AA
Yield-enhancing loci	*O. rufipogon*	QTL, *yld1*, *yld2*	AA
Yellow stemborer (larval mortality)	*O. longistaminata*	QTL	AA
Increased elongation ability	*O. rufipogon*	Unknown	AA

[a]*O. glaberrima*—African rice species. Modified from Brar and Khush [17]
[b]*O. australiensis*, *O. barthii*, *O. glumaepatula*, *O. meridionalis*, *O. officinalis*, *O. punctata*, *O. rhizomatis*. Sanchez et al. (unpublished data)

Table 2.3 Rice varieties developed through wide hybridization

Key trait	Wild species	Varieties released	Country
Grassy stunt resistance	*O. nivara*	Many rice varieties	Rice growing countries in Asia
BPH resistance	*O. officinalis*	MTL 98, MTL 103 MTL 105, MTL114	Vietnam
Acid sulfate tolerance	*O. rufipogon*	AS 996	Vietnam
Salinity tolerance	*O. rufipogon*	BRRIdhan55 (As996)	Bangladesh
Tungro resistance	*O. rufipogon*	Matatag 9	Philippines
Bacterial blight resistance	*O. longistaminata*	NSICRc 112	Philippines
Blast resistance	*O. rufipogon*	Dhanarasi	India
	O. glaberrima[a]	Yun Dao	YAAS, China
High yield, earliness, weed competitive ability, and tolerance to abiotic stresses	*O. glaberrima*[a]	Many Nerica lines/varieties	African countries
Tolerance to heat	*O. meridionalis*	Arizona Rice-1[b]	USA
		Arizona Rice-2[b]	USA

Modified from Brar and Singh [18] and Sanchez et al. (unpublished data)
[a]*O. glaberrima*—African rice species
[b]*Arizona Rice-1 and 2*—varieties to be released in 2013, Sanchez et al. (unpublished data)

Table 2.4 Rice varieties developed through MAS carrying *Xa21* gene from *O. longistaminata* and *Bph18* from *O. australiensis*

Inbreds/hybrids	Year	Resistance gene(s)	Institute/country
NSICRc 142 (Tubigan 7)	2006	*Xa4*+*Xa21*[a]	PhilRice, Philippines
NSICRc 154 (Tubigan 11)	2007	*Xa4*+*Xa21*[a]	PhilRice, Philippines
Improved Sambha Mahsuri	2007	*Xa5*+*xa13*+*Xa21*[a]	India
Improved Pusa Basmati 1	2007	*Xa5*+*xa13*+*Xa21*[a]	India
Xieyou 218	2002	*Xa21*[a]	China
Zhongyou 218	2002	*Xa21*[a]	China
Guodao 1	2002	*Xa4*+*xa5*+*xa13*+*Xa21*[a]	China
Guodao 3	2004	*Xa4*+*xa5*+*xa13*+*Xa21*[a]	China
Neizyou	2002	*Xa4*+*xa5*+*xa13*+*Xa21*[a]	China
Ilyou 8006	2005	*Xa4*+*xa5*+*xa13*+*Xa21*[a]	China
Ilyou 218	2005	*Xa21*[a]	China
ZhongbaiYou 1	2006	*Xa21*[a]	China
Suweon 523	2011	*Bph18*	Korea

Modified from Brar and Singh [18]
[a]*Xa21* gene has also been transferred into many elite inbreds and parental lines of hybrids by several institutes in India, Philippines, and Thailand

4 Examples of Crosses Between Wild AA Genome Species and Cultivated Rice

To date many chromosome segmental substitution lines (CSSLs) or BILs in rice have and are being developed and new varieties are continuously being released at different research stations around the world (Table 2.3). Crosses between cultivated rice (*O. sativa*, $2n=24$, AA) and AA genome wild species can be easily made. Hybrids between *O. sativa* and *O. rufipogon* are partially fertile; however, *O. sativa*×*O. glaberrima* and *O. sativa*×*O. longistaminata* F_1s are highly sterile. Among the classical examples are the introgression of a gene for grassy stunt virus resistance from *O. nivara* to cultivated rice

varieties [40] and the transfer of a CMS source from wild rice, *O. sativa* f. *spontanea*, to develop CMS lines for commercial hybrid rice production [44]. Other useful genes, such as *Xa21* for BB resistance, were transferred into rice from *O. longistaminata*, and new CMS sources from *O. perennis* and *O. glumaepatula*. Genes for tungro virus tolerance and tolerance to acid sulfate soil conditions have been transferred from *O. rufipogon* into *indica* rice cultivars. Some of the breeding lines with genes introgressed from wild species have been released as varieties (Table 2.3). Ram et al. [55, 56] transferred broad spectrum blast resistance from *O. rufipogon* and also released a variety, Dhanarasi. Some of the alien genes have been tagged with molecular markers and used in MAS (Table 2.4).

At Kyushu University in Japan, Yoshimura et al. [74] developed a series of introgression lines using *O. glaberrima*, *O. glumaepatula*, *O. meridionalis*, *O. nivara*, and *O. rufipogon* accessions as donor parents in *O. sativa* cv. Taichung 65 background [20, 21, 43, 46, 62, 63]. Using these introgression lines, alleles associated with a number of desirable traits were identified, such as awn character [46], days to heading [59] and seed shattering [58], and green leafhopper resistance [23, 24].

In the USA, a University of Arizona, USDA, ARS, and University of Arkansas collaborative study is developing four BIL libraries using several *O. barthii* accessions as donors and *O. sativa* cv. LaGrue and M-202 as the recurrent parents (Eizenga and Sanchez, unpublished). These introgression lines will be tested for heat and drought tolerance in Arizona. At USDA, ARS, Stuttgart, AR, under the RiceCAP program, three BIL libraries are being developed using *O. nivara* and *O. meridionalis* as donors and *O. sativa* cv. Bengal and Lemont as the recipient parents. The introgression lines will be used to map sheath blight and blast resistance genes (Eizenga, personal communication). A collaboration between Cornell University, USDA, ARS Stuttgart, AR, and the University of Arkansas developed introgression lines using three diverse *O. rufipogon*/*O. nivara* accessions as donors and *O. sativa* cv. IR64 and Cybonnet as recurrent parents [67].

At Huazhong Agricultural University in China, backcrossing programs are underway to develop 14 CSSL/IL libraries using seven AA genome *Oryza* species accessions (6 wild and 1 *O. glaberrima*) that have BAC-end sequence as part of OMAP ([69]; Y. Sibin, personal communication) as donors and *O. sativa* cv. Zhenshan 97B and 93-11 as recurrent parents. The donor wild AA genome accessions include *O. barthii*, *O. glumaepatula*, *O. meridionalis*, *O. nivara*, and *O. rufipogon*.

The CIAT/IRD (International Center for Tropical Agriculture and Institut de Recherche pour le Développement) rice genetics and genomics group lead a Generation Challenge Project (GCP) that is developing four libraries of CSSLs with the wild species *O. barthii*, *O. glumaepatula*, *O. meridionalis*, and *O. rufipogon* as donors, all sharing the same genetic background of the *tropical japonica* cultivar Curinga. The GCP-associated partners with this effort are Cornell University (USA), Fedearroz (Colombia), Embrapa-CNPAF (Brazil), and AfricaRice (Benin) (see http://www.generationcp.org/arm/ARM06/day_2/Lorieux_part_1.pdf; http://www.generationcp.org/arm/ARM06/day_2/Lorieux_part_2.pdf; [1]). Development of introgression lines from the *O. sativa* × *O. glumaepatula* interspecific cross [57] was also undertaken in the GCP initiative.

5 Introgression from *O. glaberrima* into *O. sativa*

Cultivars of Asian rice *O. sativa* are high yielding, whereas African rice, *O. glaberrima*, is low yielding. However, *O. glaberrima* has several desirable traits, such as resistance to rice yellow mottle virus (RYMV), African gall midge, and nematodes, and tolerance to drought, acidity, and iron toxicity. Another important feature of *O. glaberrima* is its strong weed competitiveness. Thus, interspecific hybridization among Asian and African species offers tremendous potential for combining the high productivity of *O. sativa* with tolerance to biotic and abiotic stresses of *O. glaberrima*. F_1 hybrids between *O. sativa* and

O. glaberrima, in spite of complete chromosome pairing, are highly sterile. Backcrossing is used to restore fertility and derive agronomically desirable lines. Molecular analysis has revealed frequent exchange of segments between *O. sativa* and *O. glaberrima*.

Efforts have been made by the Africa Rice Center (ARC) to introgress genes for weed competitiveness from *O. glaberrima* into elite breeding lines of *O. sativa* [39]. At IRRI, a large number of AILs have been produced from crosses between *O. sativa* and several *O. glaberrima* accessions. These progenies are being evaluated in collaborative projects with ARC and NARES for introgression of tolerance to RYMV, African gall midge, and abiotic stresses. Promising lines tolerant to iron toxicity in the genetic background of IR64 (IR75870-5-8-5-B-5-B), IR69502-6-SRN-3-UBN-1-(IR80340-23-B-12-6-B), and IR554230-01 (IR80314-4-B-1-3-B) have been field-tested in Iloilo, Philippines.

Bimpong [8] identified two lines (IR80311-9-B-1-2 and IR80311-2-B-1-2) derived from *O. glaberrima* showing tolerance to nematode, *Meloidogyne graminicola*, based on gall midge rating and Pf/Pi ratio. The CSSL library of MG12, an *O. glaberrima* accession, in the background of the *tropical japonica* cultivar, Caiapo, was used to identify QTLs for rice stripe necrosis virus resistance and yield components [27].

Bimpong [9] and Bimpong et al. [10] analyzed backcross progeny derived from crosses of two *indica* rice varieties (IR64 and IR55423-01) with *O. glaberrima*. The progenies were evaluated under drought stress and a number of drought-related QTLs were identified. *O. glaberrima* contributed 50–67 % of the alleles to the newly identified QTL. Two QTLs for grain yield per plant (*ypp2.1* and *ypp4.2*) were new and two others (*yld1.1* and *yld8.1*) were common in two locations. In IR55423-01 × *O. glaberrima*, 11 new QTLs for biomass were identified, of which one QTL (*bm8.1*) was common in two locations. Nine QTLs for yield, of which two were new (*ypp3.1* and *ypp8.2*), were identified. Three QTLs (*bm2*, *dth2*, and *dth4*) were common in two populations derived from IR64 and IR55423-01. QTLs *ypp6.1*, *bm6.1*, *hi6.1*, and *ps6.1* associated with an increase in grain yield were identified in the same region on chromosome 6 at locus RM275.

The University of Arizona, USDA, ARS, and the University of Arkansas collaborative project also used *O. glaberrima* as a donor to develop introgression lines with two US rice cultivars, LaGrue, a long grain *tropical japonica*, adapted to the southern region of the USA, and M-202, a medium grain *temperate japonica* adapted to California, as recurrent parents (Eizenga and Sanchez, unpublished).

6 Identification and Introgression of Yield-Enhancing Loci/QTLs

As discussed, the wild relatives of rice are phenotypically inferior to cultivated rice, with respect to agronomic traits. However, the detection of transgressive segregation for yield in crosses between cultivated and wild species suggests that, despite their inferior phenotypes, the wild relatives of rice contain genes that can improve quantitative traits, such as yield.

Using advanced introgression lines, alleles from *O. rufipogon* (IRGC 105491) were simultaneously identified, mapped, and introgressed into the genetic background of several adapted cultivars [47]. Yield-enhancing traits from this *O. rufipogon* accession were incorporated into the genetic background of IR64 [19, 60], Jefferson [65], V20B maintainer line of CMS line "V20A" [71], and Hwaeongbyeo [72]. Alleles associated with the yield-related traits, such as spikelet number, grain weight, and panicle length, were identified in this *O. rufipogon* accession using a BIL population derived from a cross with Zhenshan 97B [1]. Similarly, alleles associated with yield-related traits from two other *O. rufipogon* accessions were introgressed into TeQing [64] and Guichao 2 [66]. Yield trials of selected Jefferson/*O. rufipogon* NILs revealed yield-enhancing QTLs when compared to the donor parent [42].

QTLs from wild AA genome species for increased yield have also been identified by Xiao et al. [70]. *O. rufipogon* alleles at two marker

loci, RM5 (*yld1-1*) on chromosome 1 and RG256 on chromosome 2 (*yld2-1*), were associated with enhanced yield. In another experiment, Xiao et al. [71] identified 68 QTLs. Of these, 35 (51 %) had trait-improving alleles derived from the wild species, 19 of which had no deleterious effects on other characters.

Moncada et al. [48] and Septiningsih et al. [60] also reported QTL, "wild species alleles," as having beneficial effects for yield and yield components. Yoon et al. [73] also mapped QTL for yield components introgressed from *O. grandiglumis*. Imai et al. [32] evaluated advanced backcross progenies (BC_3, BC_4) in field trials in Arkansas and showed an average yield enhancement of 23 % compared to the recurrent parent "Jefferson." McCouch et al. [47] summarized the results of various studies on transgressive segregation and QTL responsible for increased yield from crosses of *O. sativa* × *O. rufipogon* supporting that yield-enhancing loci from wild species could increase yield potential of both inbred and hybrid rice varieties. Future research should focus on the identification of QTL from wild species and introgression into high yielding elite breeding lines.

Results at IRRI of advanced backcross progeny derived from the crosses of an elite breeding line of new plant type (NPT) rice, with *O. longistaminata* and IR64 × *O. rufipogon*, also support transgressive segregation for yield and yield components. These findings show that genes from wild *Oryza* species can increase the yield of elite rice lines, even though wild species are phenotypically inferior to cultivated rice. Yield-enhancing QTL, "wild species alleles," identified need to be transferred into high yielding genotypes and validated in well-designed field experiments.

7 Introgression for Tolerance to Abiotic Stresses

Little or no work has been done on the transfer of genes for tolerance to abiotic stresses from wild *Oryza* species into rice. Recently, the Arizona Genomics Institute (AGI) evaluated the performance of wild rice species under natural conditions in Arizona. Representative accessions of two AA genome wild rice species, *O. meridionalis* and *O. barthii*, and an EE genome *O. australiensis* were chosen from eight wild rice species grown under natural environmental conditions. The Arizona environment is characterized by limited precipitation of less than 2 mm and varying extreme temperatures (35–43 °C) during summer, where rice can be grown. Hence, Arizona is a perfect place for heat and drought studies. The three species tested showed medium to tall plant height and high tillering ability. These traits are important for developing heat- and drought-tolerant rice varieties because height and tillering of cultivated rice have been shown to decrease by at least 30 % and 20 %, respectively, when subjected to heat and drought (Sanchez and Wing, unpublished).

Two heat-tolerant varieties, *Arizona Rice-1* and *Arizona Rice-2*, were developed from a cross between one of the best accessions of *O. meridionalis* and *O. sativa* cv. M-202. These varieties were selected from advanced backcross inbred lines that resemble the *O. sativa* phenotypic traits. These varieties are currently being evaluated for heat and drought in the field. At least five additional *O. meridionalis* accessions from a total of 18 are currently crossed to *O. sativa* in order to develop more heat- and drought-tolerant varieties.

Crosses were made between *O. barthii*, *O. glaberrima*, and US cultivars to develop CSSLs and BILs. Populations generated from these crosses will be used to develop new varieties with other abiotic stress-related traits (Eizenga and Sanchez, unpublished). Most of the parental donors and recurrent parents used in developing ILs for abiotic stress have been re-sequenced using second-generation sequencing technology (Wing and Sanchez, unpublished).

IRRI evaluated several introgression lines derived from crosses between *O. sativa* × *O. rufipogon* and *O. sativa* × *O. glaberrima* at hotspots under field conditions for tolerance to abiotic stresses at Iloilo, the Philippines. Elite breeding lines with good agronomic traits and moderate tolerance to iron toxicity, aluminum toxicity, and

acid sulfate conditions have been identified. One of the wild species (*O. rufipogon*) that grows under natural conditions in the acid sulfate soils of Vietnam was used in crosses with IR64. Three promising lines were selected and tested through the yield-testing network of the Cuu Long Delta Rice Research Institute (CLRRI), Vietnam. Of the three breeding lines, IR73678-6-9-B has been released as a variety (AS996) for commercial cultivation in the Mekong Delta, Vietnam. This variety has become popular and occupies 100,000 ha (Bui Chi Buu, personal communication). It is a short-duration (95–100 days) semi-dwarf variety with good plant type suitable for moderately acid sulfate soils and is tolerant to BPH and blast. Nguyen et al. [51] mapped QTL for aluminum toxicity tolerance introgressed from *O. rufipogon* into rice.

A set of advanced introgression lines derived from *O. rufipogon* have been tested for elongation ability under deep water conditions in the Philippines and India. One of the lines in the All India Coordinated Rice Improvement Project has shown good promise.

8 Introgression of Genes from Distantly Related Genomes

Introgression lines have been produced from crosses of *O. sativa* with distantly related species with CC, BBCC, CCDD, EE, FF, GG, and HHJJ genomes. However, gene transfer has been achieved only from the CC, BBCC, CCDD, EE, and FF genomes (Table 2.2). So far, no introgression could be achieved from GG and HHJJ genomes.

Jena and Khush [37] produced several introgression lines from an O. *sativa*×*O. officinalis* cross. One of the most successful examples of the transfer of genes from the C genome wild species rice is that of brown planthopper (BPH). Four genes, *Bph10*, *Bph18*, *bph11*, and *bph12*, have been transferred from *O. officinalis* to rice. Four breeding lines have been released as varieties (MTL95, MTL98, MTL103, and MTL110) for commercial cultivation in the Mekong Delta, Vietnam. Hirabayashi et al. [28] mapped *bph11*and *bph12* introgressed from wild species on chromosomes 3 and 4. Huang et al. [30] also transferred BPH resistance from *O. officinalis* into Zhensheng 97B.

9 Introgression from the BBCC Genome Species

An advanced set of introgression lines was produced and resistance to BB and blast was transferred from *O. minuta* [2]. The introgressed blast resistance gene has been designated *Pi9*(t) and has resistance to several isolates of blast. Introgression lines were produced from a NPT×*O. minuta* cross and evaluated for resistance to 10 Philippine races of bacterial blight, where the NPT parent was susceptible to each of the 10 races. One of the families, WHDIS 1958-19, was found to have a broad spectrum of resistance to all the 10 races tested. The genes introgressed from *O. minuta* seem to have a wide spectrum of resistance, and also the number of genes introgressed could be more than one. Similarly, BPH resistance from *O. minuta* has been transferred to rice [11]. These lines have shown a wide spectrum for resistance to BPH in the Philippines and Korea.

Rahman et al. [54] conducted genetic analysis of BPH resistance using an F_2 population derived from a cross between an introgression line, "IR71033-121-15," from *O. minuta* (accession number 101141) and a susceptible Korean *japonica* variety, "Junambyeo." Two major QTLs have been designated *as Bph20*(*t*) on chromosome 4 and *Bph21*(*t*) on chromosome 12.

10 Introgression from the CCDD Genome Species

A number of breeders have produced hybrids between rice and CCDD genome species [12, 61]. Several introgression lines derived from *O. sativa*×*O. latifolia* have been evaluated for introgression of useful traits [50]. Genes for resistance to BPH and BB have been introgressed from *O. latifolia*. Yoon et al. [73] reported yield-enhancing QTLs from *O. grandiglumis*.

11 Introgression from the EE Genome Species

Multani et al. [50] produced hybrids between colchicine-induced autotetraploids of rice and *O. australiensis* ($2n=24$ EE). Introgression was detected for morphological traits, such as long awns, earliness, and the *Amp-3* and *Est-2* allozymes. Of 600 BC_2F_4 progenies, four were resistant to BPH, two (IR65482-4-136 and IR65482-7-216) of which have proven to be resistant to a Korean BPH population. One of the lines (1R65782-4-136-2-2) carried the *Bph10* gene located on chromosome 12 [34].

A major resistance gene *Bph18*(*t*) has been identified in an introgression line (IR65482-7-216-1-2) that has inherited the gene from *O. australiensis*. A marker allele of 1,078 bp completely co-segregated with the BPH resistance phenotype. STS marker 7312.T4A was validated from two temperate *japonica* backgrounds [36]. Bph18 has been transferred through MAS, and a japonica variety, Suweon 523, has been released (KK Jena, personal communication).

Jeung et al. [38] identified a new gene in the introgression line IR65482-4-136-2-2 that inherited a resistance gene from *O. australiensis* (Acc. 100882). Molecular analysis localized a major resistance gene, *Pi40*(*t*), on the short arm of chromosome 6. Following association analysis and detailed haplotyping, a DNA marker, 9871.T7E2b, was identified and found to be linked to the *Pi40*(*t*) gene at the 70 kb chromosomal region and differentiated the *Pi40*(*t*) gene from the LTH monogenic differential lines possessing genes *Piz*, *Piz-5*, *Piz-t*, and *Pi-9*. *Pi40*(*t*) was validated using the most virulent isolates from Korea as well as the Philippines, suggesting a broad spectrum for the resistance gene.

12 Introgression from the FF Genome Species

A series of introgression lines have been derived from a cross between *O. sativa* (cv. IR56) and *O. brachyantha* ($2n=24$ FF). IR56 is susceptible to bacterial blight races 1, 4, and 6 from the Philippines, whereas *O. brachyantha* is resistant. Of the 149 backcross progeny analyzed, 27 showed introgression for resistance to bacterial blight races 1, 4, and 6 [11]. Introgression for awning and growth duration has also been achieved.

13 Introgression from the GG and KKLL Genome Species

Hybrids have been produced from a cross between *O. sativa* and *O. granulata* [12]. Advanced progenies have also been produced; however, none of the lines tested have shown introgression of traits from *O. granulata* into rice.

The tetraploid *ridleyi* complex comprises two species: *O. ridleyi* and *O. longiglumis*. *O. ridleyi* shows strong resistance to all the 10 Philippine races of BB. Hybrids between rice cv. IR56 and O. *ridleyi* (accession 100821) have been produced; however, the cross shows a strong necrosis phenotype. Thus, only a few introgression lines (BC_3F_3) from this cross have been produced, but no introgression could be detected.

Intergeneric hybrids between *O. sativa* and *O. coarctata* (KKLL) have been produced through both sexual crosses following embryo rescue [13] and protoplast fusion [35]. The hybrid ($2n=36$) is sterile and shows no chromosome elimination of either parent. Due to strong incompatibility barriers, no backcross progenies could be obtained.

BC_2 progenies derived from crosses of *O. sativa* with *O. officinalis* (CC), *O. australiensis* (EE), *O. brachyantha* (FF), and *O. granulata* (GG) resembled the recurrent rice parent for most morphological traits. This suggested limited recombination between the A genome of *O. sativa* and the C, E, F, and G genomes of the wild species. Progenies recovered in BC_2 of *O. sativa* × *O. officinalis* were so similar in morphology to *O. sativa* that they were evaluated in field trials and released as varieties for commercial cultivation in Vietnam. Molecular analysis also supports introgression of small segments, limited recombination between rice and wild species chromosomes as the possible cause for the rapid recovery of the recurrent parent phenotype.

Some of the genes (e.g., *Xa21* and *Bph18*) introgressed from wild species have been used in MAS and varieties have been released (see for review [17, 18]).

14 Utilization of Wild Species for Rice Improvement: Future Priorities

- Search for new genes from diverse sources with wide spectrum of resistance in the wild species germplasm.
- Identify resistance to major biotic (sheath blight, stem borer, false smut, neck blast) and abiotic stresses (drought, heat, etc.) where there is limited variability for the target traits in the cultivated species. Transfer such genes into high yielding rice genotypes.
- Allele mining to identify novel genes/QTL with different mechanisms of resistance and pyramid such genes/QTL to enhance tolerance to major biotic and abiotic stresses.
- Identification and introgression of yield-enhancing loci "wild species alleles" into elite breeding lines to further increase the diversity and yield potential of *indica* and *japonica* rice cultivars.
- Develop CSSLs from different wild species for mapping genes/QTL and use in functional genomics; BAC libraries developed under OMAP could be used advantageously in genomics and breeding research.
- Search for genes controlling homologous pairing to promote recombination and transfer genes from distant genomes of wild species into rice.
- Intensify exploratory research on C4ness in wild species.
- Exploratory research to identify endophytes in wild species as novel source for nitrogen fixation is emphasized.
- Explore the production of haploids particularly in *indica* rice through wide hybridization similar to the existing chromosome eliminating system in wheat and barley.
- Explore the production of biofuels from wild rice with high biomass yield.
- Collect new *Oryza* germplasm accessions from different countries, with an emphasis on *Oryza* hotspots to enhance genetic diversity in rice through wide hybridization.

Wild species are an important genetic resource to broaden the gene pool of rice for tolerance to biotic and abiotic stresses. The extinction of wild species is a threat to genetic diversity and international efforts are needed to overcome the trend in the loss of biodiversity. Integration of the *Oryza* Map Alignment and *Oryza* Genome Evolution projects (OMAP, OGEP) with the wide hybridization research is emphasized to develop improved high yielding varieties with multiple resistances to biotic and abiotic stresses. With the advances in tissue culture, molecular markers, and genomics, the scope for utilization of wild species in genetic enhancement of rice seems more promising than before.

15 The Genomics of the Genus *Oryza*

As described above, tremendous progress has been made toward the introgression of important genes from the wild relatives of rice into cultivated rice using classical breeding approaches. However, in order to fully utilize and understand the genetic diversity hidden within the genus a more systematic approach must be taken using the tools of genomics.

Since 2003, the *Oryza* Map Alignment and *Oryza* Genome Evolution projects (OMAP, OGEP) have led to the establishment of a genus-level comparative genomics platform to be able to fully interrogate the genus *Oryza*. This work has led to the creation of large array of publicly available genomic resources most notably a set of manually edited BAC-based physical maps (i.e., 18 deep-coverage BAC libraries—fingerprinted, end-sequenced, and FPC assembled) representing 18 of the 24 recognized *Oryza* species, covering all 8 AA genome species and one each of the other 9 genome types (BB, CC, BBCC, CCDD, EE, FF, GG, KKLL, HHJJ) [3, 5, 6, 41]; and a set of chromosome 3 short arm sequences from all 8 AA genome species, as

well as the BB, CC, BBCC, FF, GG, and *Leerisa perrieri*, an *Oryza* outgroup species. All of these data and resources are accessible through the www.Gramene.org and www.genome.arizona web sites, respectively.

Analysis of these data sets revealed the following key points: (1) LTR Retro-transposable element amplifications dramatically increased the size of both the *O. australiensis* [EE] and *O. granulata* [GG] by as much as 400 and 200 Mb, respectively [7, 53]; (2) the AA genomes of *O. nivara*, *O. rufipogon* (the putative progenitor species of *O. sativa*), and *O. glaberrima* have expanded/contracted by at least 40 Mb (>10 % of their genome sizes) relative to the IRGSP RefSeq [31]; and (3) analysis of the *Adh1* region (~100–200 kb) across the entire *Oryza* phylogeny (diploid and polyploidy) showed significant perturbations of synteny including dynamic evolution of gene families, transposable element-mediated gene movement, mutations, genome size changes, and large scale physical rearrangements [3, 4, 6].

The overriding conclusion from these studies, and many others, indicates that a SINGLE reference genome for the genus *Oryza* (i.e., IRGSP RefSeq) is insufficient to capture and understand the allelic diversity/natural variation hidden with the genus to help solve the 9BPQ.

Table 2.5 *Oryza* reference genome sequencing project

O. sativa ssp. *japonica* [AA]	IRGSP, completed
O. sativa ssp. *indica* [AA]	BGI, completed
O. nivara [AA]	Y. Hsing, Taiwan, completed, unpublished
O. rufipogon [AA]	B. Han, China, completed, unpublished
O. glaberrima [AA]	R. Wing, USA, completed, unpublished
O. barthii [AA]	R. Wing, USA, completed, unpublished
O. longistaminata [AA]	W. Wang, BGI, PRC, completed, unpublished
O. punctata [BB]	R. Wing, USA, completed, unpublished
O. brachyantha [FF]	M. Chen, BGI, China, completed, unpublished
Leersia perrieri	R. Wing, USA, completed, unpublished
O. glumaepatula [AA]	A. Oliveria, Brazil, in progress
O. meridionalis [AA]	R. Henry, Australia, O. Panaud, France, in progress
O. officinalis [CC]	N. Kurata, Japan, in progress
O. eichingeri [CC]	N. Kurata, Japan, in progress
O. rhizomatis [CC]	N. Kurata, Japan, in progress
O. australiensis [EE]	O. Panaud, France, in progress
O. granulata [GG]	L. Gao, China, in progress

IRGSP International Rice Genome Sequencing Project, *BGI* Beijing Genomics Institute

16 The International *Oryza* Map Alignment Project

To address this resource/knowledge gap the International *Oryza* Map Alignment Project (I-OMAP) was organized and has held five grand challenge meetings (Japan 07, Korea 08, Philippines 09, Brazil 10, Taipei 11) in conjunction with the annual International Symposia on Rice Functional Genomics (ISRFG). The three primary focus areas of I-OMAP are to: (1) generate RefSeqs & Transcriptome data sets for all eight AA genome species and a representative species of the nine other genome types; (2) generate, map, and phenotype advanced ABC, CSSL, RIL populations for the AA genome species for functional and breeding studies; and (3) identify collections of naturally occurring populations of the wild *Oryza* species for diversity, conservation, population, and evolutionary analyses.

As focus Area 2 has already been discussed in detail (above), this section will focus on Area 1: the generation of RefSeqs & Transcriptome data sets for all eight AA genome species, and a representative species of the nine other genome types. Table 2.5 lists the status of each *Oryza* genome project as of October 2012. Sixteen of the 23 genome sequencing project are in progress or have been completed, and include all the diploid *Oryza* species. Draft sequences of two subspecies of *O. sativa* were published a decade [26, 75] ago followed by the IRGSP "gold standard" RefSeq of *O. sativa* ssp. *japonica* (cv. Nipponbare) in 2005 [33]. Significant progress has been achieved

over the past ~2 years with completion of the *O. glaberrima* [AA], *O. barthii* [AA], *O. longistaminata* [AA], *O. punctata* [BB], and *O. brachyantha* [FF] genomes (all unpublished but in Genbank). Assembly is currently progress for the *O. nivara* [AA], *O. rufipogon* [AA], and the *O. glumaepatula* [AA] genomes, and sequencing is underway for a majority of the remaining diploid species.

It should be noted that the I-OMAP project has a huge advantage over other next generation genome sequencing projects (e.g., *Drosophila* 12 genomes; [22]) in that physical maps are available for all AA genome species as well as representatives of all other nine genome type. Such resources facilitate the assembly of more complete genome sequences vs. ones that rely solely on next generation short-read sequence data and assembly algorithms, the so-called gene space assemblies.

These genomes are presently being comparatively annotated using a common annotation platform "MAKER" [29] and a publication is planned for the summer of 2013.

Once the I-OMAP consortia has completed the *Oryza* RefSeq project, these sequences can be used as references upon which resequencing data from the majority of all *Oryza* accessions can be mapped in order to capture the majority of allelic diversity that is present within the genus. This data in turn can be used: (1) to accelerate MAS of agriculturally important traits as described above; (2) to identify and clone new wild alleles that can be integrated into elite rice lines via genetic engineering; and (3) to conduct a plethora of evolutionary tests aimed at the identification and testing of genes that are under adaptive selection that will open new opportunities for growing rice in nontraditional rice growing areas.

Such genetic diversity work is already underway for cultivated rice but is sorely lacking for the wild relatives of rice.

17 Conclusion

As the world approaches the 9 billion people mark by the middle of this century, the rice community must do everything it can to help feed a hungry world. Rice scientists are unified in their goal to develop a wave of new designer rice varieties that are "greener," in terms of environmental impact (i.e., less water, fertilizer, pesticides, herbicides), and significant increases in yield. As outlined in this chapter, the wild relatives of rice will play an important role in the generation of the new "green super rice" varieties [76] that are required to help solve the 9BPQ.

With the advent of next generation sequencing information combined with genetic maps and molecular markers (SSRs, SNPs, SNVs), it is now possible to rapidly map and identify regions of the genome associated with specific components of a phenotype and determine which parental line contributes the favorable allele(s) at a particular locus. These are helpful tools that provide information for selecting which genes or components of quantitative trait variation to introduce from the wild gene pool into elite cultivars.

Traditional introgression approaches have been very successful in the transfer of numerous traits into cultivated rice, but have been limited due to sterility and wide crossing barriers. Using the tools of genomics we anticipate a full array of 16 reference quality *Oryza* genome sequences by the summer of 2013 at the latest. Such a data set will facilitate rapid gene discovery from the wild relatives of rice and provide the evolutionary insights needed to feed the future.

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Natural Variation and Sequencing-Based Genetics Studies

3

Xuehui Huang and Bin Han

1 Introduction

With the combined natural and artificial selections (adapting to diverse climates, seasons and soils, and influence of varied cultural practices), rice has evolved enormous diversity with a wide range of morphological, physiological, and ecological variations existing in wild rice species and landraces. It is the genomic variation that produces tremendous phenotypic differences, not only between wild and cultivated rice but also within the cultivated species. Genetic variation is the most important source for generating elite varieties. With the completion of a high-quality, map-based sequence of the rice genome for the rice cultivar Nipponbare (*Oryza sativa* ssp. *japonica*) [1] and the releases of the two draft genome sequences of *O. sativa* ssp. *indica* 93-11 and *japonica* Nipponbare [2, 3], an impressive number of sequence differences between different rice accessions has been effectively identified. Recently, the advent of the second-generation DNA sequencing technologies, including the Illumina Genome Analyzer, the Roche 454, and the ABI SOLiD instruments, has dramatically enhanced sequencing throughput with relatively low cost. These platforms enable a fast generation of millions (even billions) of sequence reads for multiple samples. The approach of whole-genome resequencing has now been used in many research areas, from genetic mapping to evolutionary analysis [4–7]. Such studies have advanced fast-forward rice genetics studies on bridging the knowledge gap between genotype and phenotype [8].

Notably, dealing with second-generation sequencing data for rice genomics studies was a challenge at the beginning. The challenge came from both the limitation of the technology and the features of the rice genome itself, which could result in errors from sequencing and alignment: (1) for second-generation sequencing, all platforms generate numerous short reads (typically 40–200 bp in length) with relatively high error rate (1–2 errors per 100 raw bases); (2) for the rice genome, there are numerous repetitive sequences (many transposable elements, including ones that are several kb in length), paralogous sequences (probably due to ancestral genomic duplications), and the genome sequences between different varieties can be quite diverse, especially for highly polymorphic regions. To address these problems, many tools and methods have been developed. Owing to these advances, second-generation sequencing is now widely used in rice, and also many other crops, for high-resolution genotyping, mutation mapping, transcriptome profiling, and epigenomics studies, although there are still some weak points for the detection of insertions and deletions (indels) of large size [9].

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2 Natural Variation in Rice: Implications from Rice Genome Data

Several years ago, the release of both the *indica* and *japonica* rice genome sequences has provided us an initial picture of intraspecific variation in rice [10–13]. Sequence comparisons between an *indica* and a *japonica* rice accession revealed an average sequence polymorphism rate of ~4 bases per 1,000 bases. Sequence variants include single nucleotide polymorphisms (SNPs), small indels, structural variations (SVs; many are related to transposable elements), and highly polymorphic regions in rice. Of these variations, SNPs are the predominant variation in number, comprising >90 % of the total number of variants. A small fraction of these variants may affect gene coding and expression and thus contribute to intraspecific phenotypic distinction. Second-generation sequencing technology enabled the generation of rice genome sequences from diverse accessions. Recently, direct resequencing of 50 accessions of cultivated and wild rice was performed to detect rice genetic diversity and genome-wide variation patterns, with a total of 6.5 million SNPs and 0.8 million small indels (1–5 bp) being identified [14]. In another study, 66 rice accessions from three taxa (22 each from *O. sativa* ssp. *indica*, *O. sativa* ssp. *japonica,* and *O. rufipogon*) were sampled for whole-genome sequencing and SNP identification [15]. The genome-wide data from various sampling and sequencing strategies provide insights into rice diversity and genomic variation. In future studies, multiple high-quality genome sequences, which can be generated by deep sequencing and de novo assembly, should be fully exploited to generate a comprehensive picture of rice genome variation. Genomic comparison of the assembled sequence will permit the identification of many complex and functional variants that directly underlie phenotypic variation. The complete assembly of multiple *Arabidopsis* genomes has demonstrated the power of this approach in the detection of novel variants and the re-annotation of gene structure [16].

Some major questions associated with the study of genetic variation in rice include (1) What are the frequencies of rare variants in rice? (2) Where did the genetic variation originate? and (3) How do variations in the genome cause the phenotypic variation of many agronomically important traits? These questions may be answered by population-scale sampling, sequencing, and analyses, and many studies are currently underway in rice to address such questions.

3 Sequencing-Based Genotyping in Rice

The rice community has constructed numerous permanent mapping populations, from crosses between cultivated and wild rice, between *indica* and *japonica* accessions, and within subspecies. These genetic populations are important resources for the identification of quantitative trait loci (QTLs) underlying a wide range of agronomic traits. The populations developed from a cross between *O. sativa* ssp. *japonica* Nipponbare and *O. sativa* ssp. *indica* Kasalath (including backcross inbred lines and chromosome segment substitution lines) have been used for cloning many QTLs (http://www.rgrc.dna.affrc.go.jp/stock.html). A recombinant inbred line (RILs) population and its coupled immortalized F2 population, developed from a cross between *indica* Minghui 63 and *indica* Zhenshan 97, enabled the genetic dissection of many yield component traits and the study of the genetic basis of heterosis in hybrids [17].

Information derived from fine mapped QTLs can help us perform further gene cloning and to breed elite varieties suitable for sustainable agriculture. Unfortunately, conventional genotyping methods using simple sequence repeats (SSRs) or restriction fragment length polymorphism (RFLP) markers are both labor and time consuming, and resultant marker densities are usually low. In rice, previously identified QTLs often spanned several megabases thereby limiting one's ability to high-resolution map and clone QTLs. To address the limitation, a high-throughput method for genotyping recombinant populations was developed utilizing whole-genome resequencing data [18]. Individual lines from the populations were simply sequenced

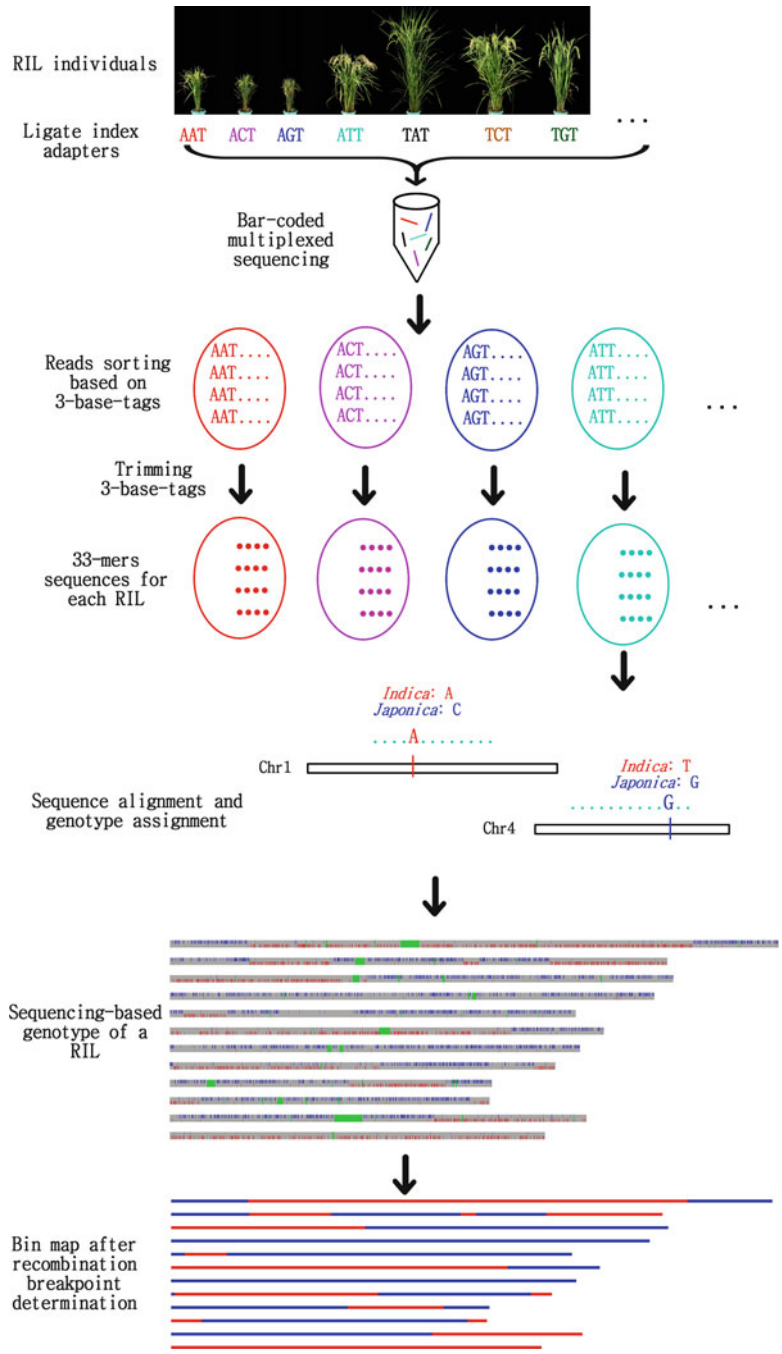

Fig. 3.1 Schematic representation of the method used for sequencing-based genotyping in rice. The population is sequenced by the second-generation sequencing to low-genome coverage per accession. A computational method implemented in the software "SEG-Map" was developed for generating a high-resolution genotype map using such sequence data

with extremely low coverage (typically ~0.02×), which still resulted in an ultra dense genotype map after several steps of computations (Fig. 3.1). The computations included SNP identification in parental lines, genotype calling and recombination breakpoint determination for each line in the

recombinant population, and recombination bin map construction. To effectively implement this method, an analytical pipeline was developed called "Sequencing Enabled Genotyping for Mapping recombination populations (SEG-Map)" [19]. SEG-Map was designed to interface with the commonly used tools for mapping second-generation short reads. The output data of SEG-Map can be used for subsequent QTL analyses directly once phenotypic data is available. This sequencing-based genotyping method is cost-effective, and the resulting genotype resolution is typically 100–1,000 times greater than using conventional methods described above. The method was firstly used in a QTL analysis of 150 RILs derived from a cross between two cultivars, *O. sativa* ssp. *indica* cv. 93-11 and *O. sativa* ssp. *japonica* cv. Nipponbare, which detected 49 QTLs for 14 agronomic traits, all high-resolution mapped (typically several hundred kbs). The method was also improved for parent-independent genotyping, that is, using the reference genome but without the availability of high-quality genotype data of the parental lines [20]. The genotyping method has been found to be generally robust for many types of mapping populations, such as RILs, near-isogenic lines (NILs), BILs, CSSLs, and F2 populations [21–23]. This method offers a powerful and cost-effective solution to map QTLs at high resolution and is now used more widely in rice and other organisms [21–24].

4 Sequencing-Based Genome-Wide Association Study in Rice

How to efficiently use the genetic diversity found in diverse rice germplasm collections for genetic mapping and breeding is an important topic. Although modern elite varieties and hybrids have achieved high yield, rice landraces maintained by farmers are endowed with tremendous genetic variability and thus have great genetic potential for rice improvement [25]. The rich variability to be exploited in landraces includes resistance alleles to drought, salinity, insects, and fungal diseases. Moreover, the wild *Oryza* resources, which are adapted to wider agroecological conditions due to long-term natural selections, have not been effectively utilized to date because of difficulties in collecting, planting, and crossing. The genetic diversity of rice germplasms accumulated in the original habitats of wild rice is becoming less and less due to the activity of modern agriculture. Many wild rice populations are extinct or near extinction. The current rice germplasm preserved across the world, including landraces and the natural populations of wild relatives, will be an extremely important resource for rice genetic improvement. So it is imminent to profile the genetic diversity of both wild and cultivated rice species for breeding elite varieties and preservation of diverse rice germplasm.

Second-generation sequencing technologies allow sequence variants to be surveyed at a much greater detail than previous approaches. Detailed analysis of a single rice accession, often coupled with whole-genome de novo assembly, typically requires deep sequencing (i.e., more than 50-fold genome coverage) and an amplification-free method for library preparation (in order to reduce the incidence of duplicate sequences). Nevertheless, when many rice accessions are sequenced, it is possible to combine low-coverage sequence data across accessions to generate accurate calls and impute missing data [26]. With a large number of rice accessions sequenced, missing calls can be inferred through computational algorithms based on linkage disequilibrium (LD) relationships between adjacent SNPs, especially for the cultivated rice accessions that have a low LD decay rate. The strategy of deploying low-coverage sequencing to large sets of phenotyped samples has important implications for GWAS, which is now a common approach for genetic mapping in human diseases [27]. The reason why a low-coverage sequencing strategy was developed is that sample sizes are important for the dissection of complex traits using GWAS and that the reduction of sequence depth means more accessions can be sequenced resulting in significant cost reductions.

The sequence-based approach was first applied in GWAS of agronomic traits using rice landraces [28]. To draw an overall picture of rice

genome variations and to develop a diverse panel for rice GWAS, an extensive collection of 1,083 rice germplasm accessions representing rice growing areas worldwide was conducted. This collection was sequenced to an approximate onefold genome coverage per accession. While the population-scale low-depth design is attractive, accompanying computational methods needed to be developed and used to maintain the quality and the integrality of the final genotype data. Onefold genome coverage results in the sampling of most genomic regions with one or two sequence reads and many regions with no reads for each individual accession. A framework for the analysis of such data was developed and included SNP identification across the population, genotype calling for low-depth sequences, and data imputation for large numbers of missing calls. The sequence data set is then used to generate a high-density haplotype map which captures most common sequence variation in cultivated rice. Using this map, a GWAS for multiple agronomically relevant traits were carried out, and many robust association signals were identified. Some known causal genes were located within the associated loci, indicating a relatively high mapping solution (approximately tens of kb). These studies provided data that can be used to gain new biological insights in rice and are helpful for rice breeders to efficiently use diverse genetic resources for rice improvement. Genomic selection, which is a new form of marker-assisted breeding selection, will probably represent a promising utility to improve cultivated rice improvement [29]. Based on large-scale phenotyping, yield-related values and grain qualities can be predicted from whole-genome SNPs data, and that knowledge can be translated to implement genomic selection [30, 31].

Major challenges for association mapping in rice include the presence of population structure and mapping resolution [32, 33]. In brief, population structure leads to complex genetic backgrounds, such as genome-wide LD between unlinked loci. Many plant species are self-fertilization which can greatly affect patterns of LD genome-wide. Several computational models have been proposed and developed to handle the confounding effects of self-fertilizing species, including principal-components analysis, mixed-linear models, multi-locus mixed models, and multi-trait mixed models [34–39]. The utility of these models can retrieve some associations for common variants with large or modest phenotypic effects. However, more improvements in experimental design are still needed [40]. For example, the dissection of agronomic traits is now also being performed using multiparent genetic mapping populations, such as the nested association mapping (NAM) population in maize, which can be an alternative method for GWAS. Future studies may use genetic populations that combine the collections of rice germplasm and recombination populations from carefully designed crosses. These efforts will improve our ability to connect complex traits with genomic variation.

Most GWAS in human offer single-gene-level mapping resolution due to the large number of historical recombination events. However, LD decays slowly in cultivated rice as mentioned above. Associated loci in rice often consist of multiple genes, so follow-up analysis is essential to detect the causal genes. Gene annotation information, expression profiles, and comprehensive sequence variant data in the local region can provide important clues for narrowing down candidate genes/regions [41, 42]. In rice, a large amount of microarray data representing transcriptomes of multiple organs in different conditions has been made publicly available. The analysis of these data made possible the understanding of important gene expression profiles and the study of their patterns, which can provide a huge amount of valuable information and accelerate discovery of unknown gene functions in determining candidate genes in associated loci of GWAS. Sequence variants in the associated loci can be detected by several ways. PCR amplification and sequencing is the most common way for the identification of potential causal variants in follow-up analyses of GWAS. It is now possible to use second-generation sequencing to detect the variants. Individual-scale deep sequencing and whole-genome assembly is the best way for identification, but the cost is relatively high.

Haplotype-based local assembly was also developed for population-scale low-coverage sequencing. These resources should be integrated together for the community, and a comprehensive database linking all the information (like TAIR in *Arabidopsis*) is needed for coordinated effort in rice functional genomics [43]. Moreover, NILs from appropriate crosses can also be used for fine mapping. T-DNA mutants and genetic transformation can be used at this stage to validate the function of candidate genes [44–46].

5 Identification of Selected Loci and Favorable Alleles in Rice Domestication and Modern Breeding

Population-scale resequencing and genome data analysis in rice has implications for understanding the genetic basis of rice domestication and subsequent improvement. The patterns of genomic variation can facilitate genome-wide selection screening to identify genes that were selected by humans. Population genetics approaches have the potential to identify loci that are subjected to domestication/breeding selection over a long period of time. There exists reduction or elimination of variation around the genomic region of a selected gene due to recent and strong human selection, which is called a selective sweep. Several methods have been developed to detect the selective sweeps, by measuring LD, allele frequency, or sequence diversity. Nowadays, the second-generation sequencing data and the resultant polymorphism data have been used to perform selective sweep screening in rice and many other organisms [14, 15]. By carefully collecting and sampling of elite varieties, traditional landraces, and wild relatives (*O. rufipogon*), it is possible to comprehensively evaluate the evidence of selection across the rice genome and discover the loci that played important roles in rice domestication (comparing genomes of traditional landraces and wild relatives) and improvement (comparing genomes of elite varieties and traditional landraces).

The selected loci may be related to a wide range of agronomically important traits, including most yield-related traits. High-density QTL mapping information and GWAS results can be used to link the loci with detailed biological function. Mapping populations between elite varieties and wild relatives may be important resources for fine mapping domestication-related or improvement-related QTLs. As domestication/improvement-related traits (e.g., seed dormancy, ripening rate, perennial/annual habit, and panicle structure) are focused by the community with more functional clues (e.g., transcriptome information of wild rice in various conditions and construction of wild rice mutant collections) [47], the causal genes associated with traits will be captured and validated.

To investigate the genetic potential of wild rice, a project entitled the "Oryza Map Alignment Project" (OMAP) was carried out with the ultimate goal of constructing and aligning BAC-/STC-based physical maps of 11 wild and one cultivated rice species to the International Rice Genome Sequencing Project's finished reference genome—*O. sativa* ssp. *japonica* c.v. Nipponbare [48]. Resequencing of the wild species of the genus Oryza offers enormous potential to make a significant impact on agricultural productivity of the cultivated rice species *O. sativa*. In order to understand the molecular genetic basis of rice domestication processes and the origin of cultivated rice, the global wild and cultivated rice resources have been exploited to generate a comprehensive picture of genomic variation [49]. The resultant high-resolution haplotype map enabled to identify the detailed genetic and geographical origin of rice. In the search of signature of domestication by adopting an integrated genomics approach, 55 selective sweeps were detected in rice genome. It is revealed that *japonica* rice was first domesticated from a subpopulation of wild rice *O. rufipogon* in Southern China and was subsequently crossed to local wild rice in south Asia and Southeast Asia thus generating *indica* rice [49].

6 Conclusions and Perspective

The tremendous rice genetic diversity has provided great resources for rice genetic studies. High-throughput sequencing technology has

promoted the detection of genetic variations in rice populations. Thus, sequencing-based rice genetics have formed a solid platform for rice functional genomics studies. Rice genes contributing to past domestication and improvement events can further guide current breeding efforts as well. In fact, loci carrying advantageous agronomic traits from Asian cultivated rice are being introduced into gene pools of African cultivated rice, combing the yield potential of *O. sativa* with stress-tolerance features of *O. glaberrima* [50]. Moreover, domestication selection and modern breeding was often accompanied with loss of diversity, especially in the *japonica* subspecies. Rice breeders should consider a careful reintroduction of useful alleles from genetic resources found in landraces and the wild rice relatives, which can be aided by the results from rice genetics studies.

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Genome Mapping, Markers and QTLs

4

Bi Wu, Zhongmin Han, and Yongzhong Xing

1 Introduction

Rice is the staple food of one-half of the population in the world. Accordingly, considerable attention has been paid to understanding the genetics and genomics of this important crop. Rice has several wild relatives and a rich supply of cultivar germplasm. Exploitation of the beneficial alleles from both cultivars and wild rice species will be critical to efficient genetic improvement of rice. The majority of important agronomic traits are quantitatively inherited in rice. Many quantitative trait loci (QTLs) regulate trait phenotypes in concert, which introduces complexities in genetic analyses. With the advent of molecular markers, many genetic linkage maps have been the constructed with different types of populations. Additionally, many statistical methods have been created to detect the QTLs that underlie the target traits. To date, QTL mapping is regarded as a powerful way to analyze quantitative traits. Mapping of rice QTLs has progressed considerably since 1990. Thousands of QTLs have been reported for yield-related traits and stress tolerance in rice. Some important QTLs have been cloned on the basis of primary QTL results. Compared with mutant-based gene discovery, the favorable alleles of QTLs detected on the basis of natural variation can be directly explored in rice breeding through the use of marker-aided selection. The markers linked to target QTLs and functional markers derived from well-characterized genes/QTLs are ideal tools for directional selection owing to their stability and low cost. This chapter will review the progress of rice markers and QTLs over the last 2 decades and evaluate the prospects for the genetic improvement of rice.

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2 Mapping Populations

Rice mapping populations are mainly classified as being derived from either biparental or multiparental populations. Biparental populations for linkage analysis are usually created from F_1 plants that are derived from two phenotypically diverse parents. Multiparental populations are collected from natural populations for linkage disequilibrium analysis, which aims to capture as many alleles of each gene pool as possible. Over the last 2 decades, many mapping populations were developed, including primary populations, secondary populations, and natural populations. The workflow developing each kind of population is summarized in Fig. 4.1.

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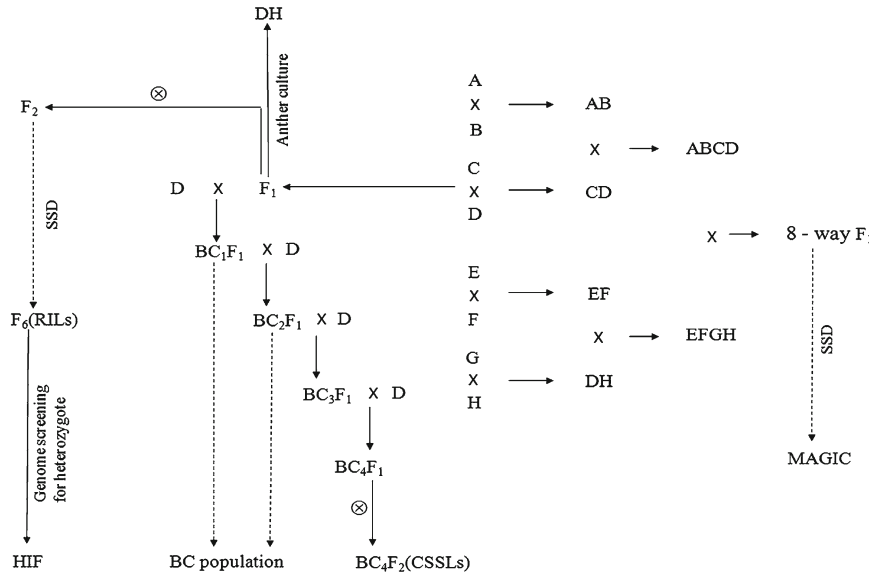

Fig. 4.1 Flow chart describing the development of mapping populations. A–H represent eight accessions, *DH* doubled haploid, *RIL* recombinant inbred lines, *HIF* heterogeneous inbred family, *BC* backcross population, *CSSL* chromosome segment substitution line, *MAGIC* multiparent advanced generation intercross population, *SSD* single-seed descent

2.1 Parents Used to Generate Mapping Populations

A segregating population is a prerequisite for mapping of either genes or QTLs. In order to enhance the power of QTL mapping, more attention should be paid to the parents for mapping population. In theory, the accessions with large genetic divergence are recommended as the parents to develop the mapping population. However, at the beginning of QTL mapping, the genetic relationship between cultivars is not clear for most germplasms owing to the availability of limited DNA sequence information. Accessions with varied trait performance are preferred when making crosses for population development [1]. For example, for QTL mapping to improve rice grain yield, the cultivars Zhenshan 97 and Minghui 63, which differ in the number of spikelets per panicle and grain weight, were chosen to make mapping populations of F_2 and recombinant inbred lines (RILs) [2–4]. The series of QTLs identified in these populations indicated the efficiency of parent selection on the basis of trait performance. In contrast, the RIL population derived from the cross between the parents Chuan 7 and Nanyangzhan, which have contrasting grain lengths, detected only two QTLs for grain length [5]. In addition, two cultivars with similar trait values, Teqing and Minghui 63, were used to develop a RIL population for yield-related QTL mapping. Transgressive segregation for many traits was observed in the population, and many QTLs were detected [6]. Obviously, all these cases indicated that parent selection on the basis of trait performance is not always efficient for powerful QTL mapping. Actually, there are three patterns of allele distributions in parents for a given target trait. The first pattern is that alleles with positive and negative effects at all or most QTLs are from one parent. In this case, target traits frequently differ substantially between parents. Many QTLs are expected in the population derived from such kind of parents. The second pattern involves segregation of only a few QTLs (probably one major and few minor effect QTLs) in parents, even though the phenotypes of both parents differ substantially, meaning that only a few QTLs could be detected in the population from the parents. The third pattern involves

random distribution of positive alleles between the two parents. In this case, although the phenotypes of both parents do not differ significantly, many QTLs are expected in the population from the parents. These three patterns correspond with the three populations mentioned above. In addition, more QTLs were frequently detected with the population derived from the cross between the cultivar and the wild rice strain compared to that between two cultivars [7–9]. Hence, genetic diversity between rice materials is a good parameter for selecting parents to produce mapping population. On the other hand, genetically diverse parents always have a high degree of DNA polymorphism, which is the major factor required for assembly of a high-density genetic linkage map. The sharply decreased cost of sequencing in recent years has enabled genome-wide analysis of the genetic diversity of candidate parents before mapping populations are developed. Thus, combined consideration of trait performance and genetic diversity is able to select parents for mapping populations in which more QTLs for target traits can be detected.

2.2 Primary Mapping Populations

The F_2, backcross (BC), and RIL populations are the three primary types of mapping populations that are widely used for QTL mapping. Primary mapping populations are classified into temporary and permanent ones according to the genetic makeup of individuals in the population. Whereas individuals in temporary F_2 or BC populations are not pure lines, individuals in permanent populations of RILs are pure lines. An F_2 population is developed by selfing an F_1 individual derived from two parents. The F_2 population is the most informative population for genetic mapping analysis [10]. This is because an F_2 individual is the result of two recombinants, the male and female gametes. It is suitable for genetic analysis. However, any F_2 individual cannot copy itself owing to its unique heterozygous genetic makeup. Thus, F_2 phenotypic data obtained from only one individual is not reliable because of environmental effects. In order to resolve the problem, F_2 phenotypic values are often represented by its derivative F_3 for QTL mapping. BC populations are developed by crossing the F_1 with one of the two parents used in the initial cross. The major drawback of using F_2 or backcross populations is the difficulty in conducting replicated experiments. A range of RIL populations are developed by single-seed descent from individual plants of an F_2 population. Importantly, each RIL is fixed for many recombination events and can self to produce a large quantity of seeds with the same genotype. A RIL population can be genotyped once and phenotyped repeatedly in several environments, both in different locations and during different years. Therefore, although the development of RIL populations is time consuming (taking 3–4 years on average), they have been widely used for QTL mapping since the end of the last century. A doubled haploid is a genotype formed when haploid cells undergo chromosome doubling. Artificial production of doubled haploids is important in rice genetics and breeding. Doubled haploid lines (DHs) are developed by culturing anthers of F_1 plant. Thus, DHs are permanent population like RILs. In terms of genetic analysis, DHs have the same advantages as RILs. However, application of DHs to genetic analysis is restricted by the genotype dependency of DH production. Both RIL and DH populations can only estimate additive effects rather than the dominant effects, which are regarded as an important factor that contributes to heterosis.

2.3 Secondary Mapping Populations

A secondary mapping population, which is also known as an advanced population, is a kind of idea population for QTL mapping. It includes near isogenetic lines (NILs) and chromosome segment substitution lines (CSSLs). The most important character of advanced populations is that segregation occurs in a local region but not in most chromosomal regions. Whereas a NIL focuses exclusively on a target QTL region, each CSSL covers a small part of the whole genome with donor parental chromosomal fragment, and

a set of CSSLs together cover the whole genome. Several NILs can be developed by screening the residual heterozygous line from advanced selfing generation according to the marker genotypes without any QTL information. Moreover, NILs for target QTLs are frequently obtained by screening inbred lines that carry heterogeneous regions from RILs used for QTL mapping. The notable particularity in these populations is that only the target chromosome segment is heterozygous. Therefore, any QTL in the heterozygous region can be interpreted as a single Mendelian factor capable of reducing the impact of background on the mapping results [11, 12].

2.4 Natural Populations

Collecting natural varieties for association mapping is regarded as a good approach to identify QTLs or genes and may identify the effects of different alleles [13, 14]. Collection with a wide variation and wide geographic distribution is the most important principle for QTL mapping. In these populations, historical recombination events persist among the selected accessions due to the common ancestry/evolutionary history. These populations have the potential to improve resolution for association analysis because they have captured the historical recombination events, which have probably accumulated over the course of hundreds of events [15–17]. However, the structure of natural population often complicates association mapping, resulting in spurious associations [16].

2.5 MAGIC Populations

Multiparent advanced generation intercross (MAGIC) populations have been used to map QTLs in mice [18, 19] and *Arabidopsis* [20]. Multiple parents are used to construct a MAGIC population. Firstly, a pair of parents is crossed to produce 2-way hybrids, the 2-way hybrids are then crossed with each other to produce 4-way hybrids, and 8-way and 16-way hybrids are generated accordingly. Thus, each multiparent hybrid carries the genetic information of all parents. Finally, the multiparent hybrids produce an F_2 population, which can be used for QTL mapping or to produce a RIL population through single-seed descent (Fig. 4.1). A MAGIC population can overcome the restricted allelic variation of the simple synthetic populations created from a cross between two parents. Meanwhile, intercrossing different combinations of multiple parents and following additional generations of self-crossing in the process of population development eliminate population structure. Therefore, a MAGIC population includes the advantages offered by the biparental and natural populations used in QTL mapping. Both linkage and association methods are suitable for the population. Future efforts at QTL mapping should consider the use of MAGIC populations.

3 Molecular Markers

Classical genetic studies cannot dissect the genetic basis of quantitative traits at the single-QTL level because no tools are available to genotype individuals on a genome-wide scale at that time. The advent of DNA markers, which are able to reveal the genome-wide difference between individuals or species, provided a considerable boost to QTL research. Three generations of markers have been born. Whereas first-generation markers are restriction enzyme-based markers, such as restriction fragment length polymorphisms (RFLP), second-generation markers consist of polymerase chain reaction (PCR)-based markers, such as random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers. All these DNA markers are developed on the basis of DNA fragment length polymorphisms or single-nucleotide mutation, which are identified by PCR or southern hybridization. Third-generation markers include single-nucleotide polymorphisms (SNP) identified by sequencing or the use of SNP arrays.

The RFLP approach reflects the variations on the DNA sequences, which can be digested by particular restriction enzymes. The three main steps involved in the long and complex process of

RFLP genotyping include (1) digestion of total DNA with a restriction enzyme(s), (2) separation of the digested DNA by agarose gel electrophoresis and transfer of the separated fragments to a solid vector nylon membrane, and (3) hybridization of the separated DNA on the nylon membrane to a labeled probe to reveal a "map" of the DNA segment. Compared with morphological markers, RFLP markers provide more genetic information because of their codominant nature [21].

The RAPD approach amplifies DNA product by using a single 10-bp primer rather than the pair of primers used in conventional PCR. The four key steps used in RAPD genotyping include (1) isolation of DNA (only nanogram amounts are needed), (2) PCR-mediated amplification using a random primer, (3) separation of DNA fragments by gel electrophoresis, and (4) visualization of the profile following staining with ethidium bromide [22]. Use of an arbitrarily selected primer permits amplification of a set of randomly distributed loci from any genome, making it easy to generate a large number of genetic markers without the need for prior information about the rice genome sequence. When such DNA sequence information was not at hand during the early stages of rice genome sequencing, RAPD markers played important roles in genetic analysis. Nonetheless, the dominant nature of RAPD markers decreased the quality of genotype data. With the completion of rice whole genome sequence, RAPD markers have gradually been replaced by SSR markers, which have codominant features, like RFLP markers.

The SSR markers consist of repeated core sequences, which have been referred to as microsatellites [23]. The tandem-repeated microsatellites are flanked by conserved sites recognized by restriction endonucleases. Thus, the length of the restriction fragment produced by this type of genetic locus is proportional to the number of core units it contains [24]. In addition, primers designed on the conserved flanking regions of simple repeat sequence amplify the entire length of SSR loci. Thus, polymorphisms in SSR lengths and RFLP markers are informative genetic markers, able to detect up to 25 alleles at some loci [25].

Single-sequence repeats are ubiquitous in eukaryotics genomes. Sequencing of the *japonica* cultivar Nipponbare [26, 27] and the *indica* cultivar 93-11 [28] has increased the convenience for developing SSR markers. A draft sequence of the genome of the *japonica* cultivar Nipponbare revealed 48,351 SSRs, which is equivalent to about one SSR every 8,000 bp [29]. The proportions of di-, tri-, and tetranucleotide SSRs in this genome are 24 %, 59 %, and 17 %, respectively. The most abundant dinucleotide SSR is the AG/CT variant, and CGG/CCG is the most frequently found trinucleotide repeat unit, representing 44 % of all trinucleotide SSRs. The most common tetranucleotide SSR is ATCG/CGAT. McCouch et al. [30] developed and validated 2,240 new SSR markers based on the 6,655 SSR-containing sequences in rice. Among the 12 most abundant motifs of the 2,240 SSR markers, poly(GA) motifs, poly(AT) motifs, and poly(CCG) motifs account for 36 %, 15 %, and 8 % of all motifs, respectively. The ratio of trinucleotides to dinucleotide content was 2.33, 0.50, and 0.18 in cDNAs; assembled contigs; and fully masked reads, respectively. These data correspond with the predicted gene content of triplet codons. These trinucleotides contain high GC contents, which is correlated with the high GC content of many rice exons [28].

Genotyping that involves the use of SSRs is faster and more economic than RFLP genotyping. Less starting DNA is required for PCR markers than for RFLP markers. Hence, SSR markers are currently the predominant genetic markers used to map rice QTLs.

After SSRs, SNPs are the next most efficient class of molecular markers. Comparative sequencing generates large amounts of DNA sequence data, which facilitates the discovery of SNP markers at the genome-wide level. SNP markers often detect two alleles at one site, but there are abundant SNPs on the whole genome. There are 1,703,176 SNPs that can be used to discriminate between Nipponbare and 93-11, which is equivalent to one SNP every 268 bp on average across the entire rice genome [31]. Feltus et al. [32] estimated approximately 1.7 SNP per 1 kb of genomic DNA, which was able

Table 4.1 Genetic characters and genotyping methods of different DNA markers and their costs in obtaining data points

Markers	Separating method	Genotype detection	Codominant	Ease of use	Cost[a] (USD)
RFLP	Southern blot	Digoxigenin, isotope	Yes	Labor intensive	43.7
SSR	PAGE (4 %)	Silver staining	Yes	Easy	8.5
RAPD	Agarose gel (1.5 %)	EB staining	No	Easy	4.5
SNP	Array or sequencing	Fluorescence, luminescence	Yes	Medium	15.8

[a]Cost was calculated on the basis of the expense genotyping 200 samples by one maker in the National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University

to discriminate between Nipponbare and 93-11. Besides SNPs, comparison of whole genome sequence also identified numerous insertions/deletions (InDels). Long InDels are very suitable for marker development, as they can be resolved using agarose gel electrophoresis. A database for InDels between Nipponbare and 93-11 was constructed, containing 479,406 InDels, which is equivalent to one InDel every 953 bp [31].

There are several prevailing SNP detection platforms, such as Affymetrix, Illumina GoldenGate, Sanger sequencing, and Illumina BeadXpress. An array with 384 SNPs was developed using the Illumina BeadXpress platform to evaluate the variation between *japonica* and *O. rufipogon* [33]. An array designed using the Illumina GoldenGate platform can detect variation within Japanese temperate *japonica* cultivars [34] and capture variation within and between subpopulations of *O. sativa* [35]. Data obtained using Sanger sequencing was used to design an array with 4,357 SNPs in order to evaluate the diversity of 1,578 genes distributed throughout the genome [36]. To evaluate diversity within and between subpopulations of *O. sativa*, a 44,100-SNP loading array was designed using the Affymetrix platform [37]. For the purpose of detecting diversity within and between *O. sativa*, *O. rufipogon*/*O. nivara*, *O. glaberrima*, and *O. barthii*, a genome-wide SNP assay was developed based on Affymetrix platform [38]. Large-scale scanning using an SNP chip would greatly enhance genotyping efficiency. Besides the use of SNP arrays, re-sequencing also enables sequence variation to be captured. New sequencing technologies have greatly decreased the cost of SNP genotyping. People prefer to genotype mapping populations through sequencing. Huang et al. [39–41] re-sequenced hundreds of rice accessions and got millions of SNPs for genome-wide association analysis.

Another approach to SNP detection involves identification of cleavage amplification polymorphic sequence (CAPS) markers. The scoring of CAPS markers depends on the variation in the sizes of fragments after digestion of the PCR product by a cocktail of restriction enzymes. For well-characterized genes, CAPS designed following causal mutation of one gene could be functional markers that identify functional alleles from natural resources. For example, a CAPS maker was designed on the causal SNP mutation of a major grain shape QTL *GS3* to identify alleles for long and short grains [42].

Low-cost and high-throughput marker genotyping system is popular in the genetic analysis. We compared the costs of genotype data at one locus for one population of 200 samples identified by different kinds of markers (Table 4.1). At 45 dollars (USD) for each marker developed, the development of RFLP markers is relatively expensive. Although the development of RAPD markers is cheap (about 4.5 dollars (USD) per marker), the popularity of RAPD markers for use in genetic analysis is limited by their dominant nature and poor repeatability. The cost of each SSR marker is about 8.5 dollars (USD), which is acceptable to most researchers. The price of developing a SNP assay depends on the number of SNPs loaded on the chip. The more SNPs carried on a chip, the cheaper the cost associated with each data point. Next-generation sequencing

promises to greatly decrease sequencing costs. Direct sequencing is becoming the most popular way to genotype SNPs, and all indications are that SNP markers will be the main tool for the foreseeable future, especially for large-scale genotyping, with SSR markers supplementing efforts for genotyping in the coming years.

4 Linkage Maps

A linkage map is a map that shows the relative positions of genes or markers on a chromosome, as determined using linkage analysis. Since 1988, different populations and markers have been used to construct rice linkage maps. Depending on the type of markers assigned to the map, there are RFLP maps, RFLP and SSR combined maps, SSR maps, and SNP maps (Table 4.2).

The first rice RFLP maps were constructed using F_2 populations [43]. After that, RFLP maps that had been developed using RILs and DHs were widely used for QTL mapping of important agronomic traits in rice [44–48]. Disease-resistance QTLs were also identified using RFLP mapping [49–51], as were insect-resistance QTLs [52] and marker-assisted selection for molecular breeding [53]. A high-density RFLP linkage map that covers 1,521.6 cM was constructed using an F_2 population from the *japonica/indica* cross [54], where 2,275 RFLP markers were assigned. Although the average marker density is high, the markers are not evenly distributed through the whole genome. There are 39 gaps larger than 5 cM.

As early as the 1990s, SSR markers were used to construct genetic maps in human [55, 56], soybean [24], and other plants [57–61]. Wu and Tanksley [62] firstly characterized ten dinucleotide microsatellites in rice and assigned them to the previous RFLP maps. They found that alleles of certain microsatellite loci are specific to the two cultivated subspecies, *indica* and *japonica*. McCouch et al. [30] developed 2,240 new SSR markers for rice, with 92 % of the primer pairs recognizing regions that encode flanking perfect repeats more than 24 bp in length. Complete sequencing of the rice genome has provided more sequence information to develop SSR markers. However, SSR markers are not evenly distributed throughout the rice genome [63, 64]. Combined application of SSR and RFLP markers can improve the resolution with which SSR markers can be mapped. Xing et al. [4] used both SSR and RFLP markers to construct the genetic map with evenly distributed markers along chromosomes. An InDel marker genetic linkage map was developed in rice [31], and PCR analysis indicated that 90 % of InDels in this database could be used as molecular markers, with 68–89 % of these markers being polymorphic with other *indica* and *japonica* subspecies. This suggests that the InDel markers identified by comparing the sequences of 93-11 and Nipponbare can be further used for analysis of other interspecies.

Next-generation sequencing has made genotyping and genetic mapping both quick and cheap. Identifying SNPs through sequencing for map construction introduces two aspects that differ from the situation with traditional genetic markers. One is that it is often not the case that all members of a RIL population can be scored at a given SNP site. The other is that an individual SNP site is no longer a reliable marker or locus

Table 4.2 The populations for genetic linkage map construction using different DNA markers

Marker	Population	Number	Size	References
RFLP	Nipponbare/Kasalath F_2	2,275	186	Harushima et al. [54]
RFLP	Lemont/Teqing F_2	113	255	Li et al. [48]
RFLP	Zhaiyeqing 8/Jingxi 17 DH	137	132	Lu et al. [46]
SSR	IR64/IRGC 105491 BC_2F_2	165	285	Septiningsih et al. [96]
RFLP/SSR	Zhenshan 97/Minghui 63 RIL	220	240	Xing et al. [4]
SNP	Nipponbare/93-11 RIL	170 K	150	Huang et al. [39]
SNP	Zhenshan 97/Minghui 63 RIL	11,792	238	Xie et al. [66]

Number marker number, *Size* population size

for genotyping owing to sequence errors. A "bin" is a position on the genetic map with a unique segregation pattern and is separated from adjacent bins by a single recombination event [65]. Huang et al. [39] developed a strategy for high-throughput genotyping of RILs derived from Nipponbare and 93-11 and constructed a bin map that spans 1,539.5 cM. A single recombination bin on average covered an approximately 100-kb interval. Xie et al. [66] created a method based on the principle of maximum parsimony of recombination to infer parental genotypes from a low-coverage re-sequencing (0.05× genome sequence for each line) of their RIL population. An ultrahigh-density linkage map was then constructed. A single bin map covered 208.5 kb for each RIL on average, in which the major grain width QTL *GW5* was mapped to within 200 kb. This study suggested that one population could be genotyped in a single run for construction of a genetic linkage map.

4.1 Physical Map

A physical map indicates the relative locations of identifiable landmarks on DNA regardless of inheritance. Distance is measured in base pairs. A physical map is a prerequisite for genome-wide sequencing of large genomes [67]. In the 1990s, bacterial artificial chromosome (BAC)-based physical maps, yeast artificial chromosome (YAC)-based maps, and STS markers were widely used for physical mapping of the entire genome of rice [67–69].

4.2 Segregation Distortion

Segregation distortion, which occurs when the observed proportion in a population deviates from the expected Mendelian segregation ratio of markers or genes, is a strong evolutionary force [70]. Segregation distortion is common in plants, such as Arabidopsis [71], maize [72, 73], wheat [74], soybean [75], and rice [76, 77]. Segregation distortion of genes and markers is frequently observed in populations derived from interspecific and inter-subspecific (i.e., *indica-japonica*) crosses of rice (*Oryza sativa* L.). The skewed segregation chromosome segments are called segregation distortion loci (SDL) [78, 79]. Lin et al. [76] used markers to identify segregation distortion in *indica-japonica* hybrids and found that markers on chromosomes 3, 7, 8, 11, and 12 showed clear segregation distortion. Harushima et al. [80] used an inter-subspecific cross F_2 population between Nipponbare and Kasalath to identify 11 major SDL on chromosomes 1, 3, 6, 8, 9, and 10 (Fig. 4.2). The strongest segregation distortion occurred on chromosome 3, with the Kasalath genotype on this position showing a 95 % probability of transmission to the progeny through pollen. Xu et al. [77] used molecular markers to identify segregation distortion in one backcross inter-subspecies population and five inter-subspecies populations, which include two F_2 populations, two DHs, and one RIL. They identified a total of 17 SDL, which distributed on all 12 chromosomes. Eight of them were on the same chromosome regions as those identified previously (Fig. 4.2).

Most SDL arise as a consequence of gamete selection and zygotic selection [81]. Chromosome segments bearing SDL are overrepresented in a population. In rice, skewed segregation was observed in the *waxy* region, which harbors a QTL for amylase content on chromosome 6. The SDL was linked to the *S5* locus, which affects the embryo-sac sterility [82]. The sterility genes identified by QTL mapping also localized to the SDL region, indicating that segregation distortion is accompanied by gamete sterility.

4.3 Recombination Suppression and Recombination Hot Spots

Recombination suppression greatly limits the mapping resolution, even for a large population. Centromeres are often associated with a depression of meiotic recombination adjacent to the pericentromeric regions [83]. For example, severe recombination suppression was observed in the *SSP7* interval within the pericentromeric region on rice chromosome 7 [84]. The ratio of

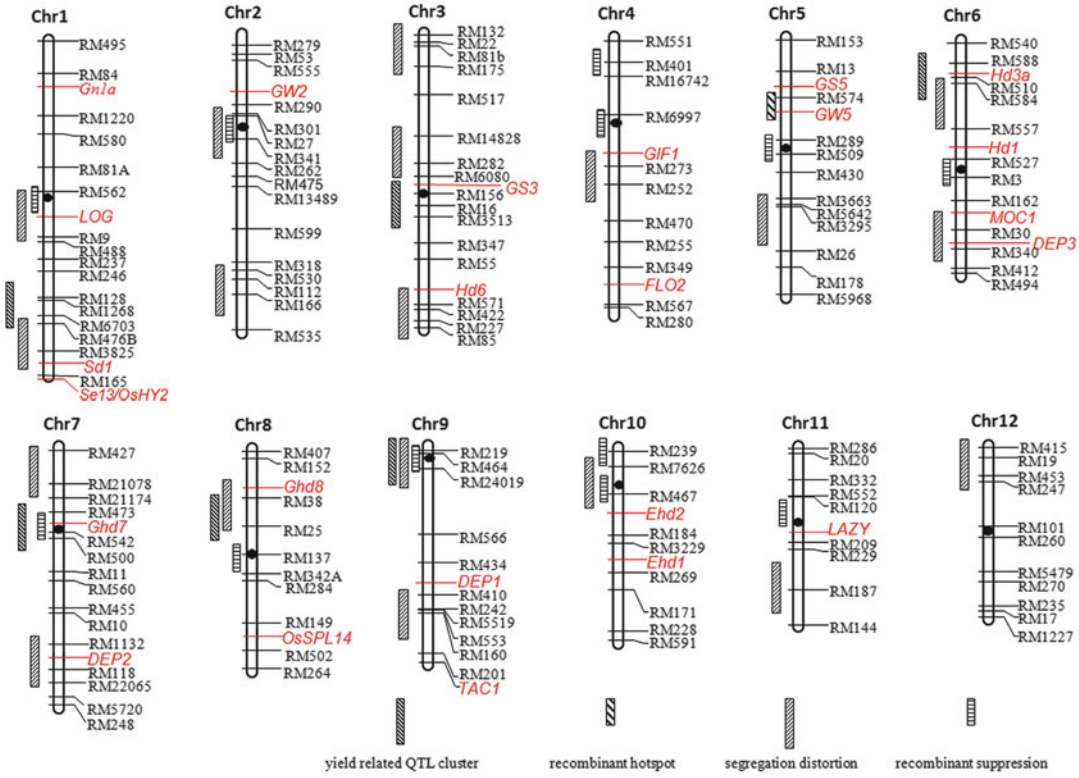

Fig. 4.2 Genetic linkage map showing the cloned genes that control yield-related traits and the regions that express recombination hot spots, cold spots, and segregation distortion

physical-to-genetic distance in this targeted region was about 6,000 kb/cM, which is 20 times higher than the estimated average ratio (250–300 kb/cM) [85]. Comparison of the genetic and the physical maps revealed that recombination is suppressed in the centromeric regions as well as the short arms of the chromosomes 4 and 10 [86]. However, no recombination suppression was reported in the pericentromeric region on rice chromosome 3 [87–89]. Recombination hot spots are small regions in the genomes of sexually reproducing organisms that exhibit highly elevated rates of meiotic recombination. The interest in recombination hot spots comes from their possible effects on the patterns of DNA sequence variation, which would be associated with trait performance. Thus, it is possible to use map-based cloning to narrow down the location of a QTL or gene in a recombination hot spot region when using an intermediate-sized population that comprises several hundred individuals. The reported recombination hot spot and cold spot regions are indicated in Fig. 4.2.

5 QTL Mapping by Linkage Analysis

5.1 QTLs for Grain Yield Traits

In rice, QTL mapping has been conducted for most agronomic traits and tolerance of biotic and abiotic stresses. For example, on the web site "gramene" (http://www.gramene.org/), there are 239 QTLs for spikelets per panicle, 214 QTLs for tillers per plant, and 316 QTLs for grain weight. Populations for QTL mapping were developed from the crosses of intra-subspecies, inter-subspecies, or wild rice cultivars. We selected eight well-studied populations to serve

Table 4.3 The number of yield-related QTLs identified in the well-studied populations

Cross	Population	QTL number				References
		Yield	GPP	TP	GW	
Ind/Ind	ZS97/MH63 F_2	5/6 (1)	5/7 (4)	3/2 (1)	7/9 (6)	Yu et al. [2]
	ZS97/MH63 RIL	3/4 (2)	3/5 (3)	3/4 (1)	7/6 (4)	Xing et al. [4]
Ind/Jap	9024/LH422 RIL	2	3	1	3	Xiao et al. [47]
	93-11/Nip. RIL		4	1	4	Wang et al. [95]
	Lemont/Teqing F_2	4	6		9	Li et al. [98]
	ZYQ8/JX17 DH		2/2/2 (2)		5/5/3(2)	Lu et al. [94]
Ind/Ruf	IR64/*O. ruf.* BC	3	2	6	5	Septintingsih et al. [96]
Jap/Ruf	Caiapo/*O. ruf.* BC	2		2	5	Moncada et al. [9]

The left and right to slash is the number of QTLs detected in different environments, and the number in the bracket is the QTL commonly detected across different environments
ZS97 Zhenshan 97, *MH63* Minghui 63, *Nip* Nipponbare, *ZYQ8* Zhaiyeqing 8, *O. ruf. O. rufipogon*

as examples that exemplify the general features of QTL mapping (Table 4.3).

Intra-subspecies hybrid-derived populations include those from *indica*/*indica* and *japonica*/*japonica* crosses. *Indica* cultivars Zhenshan 97 and Minghui 63 are the parents of the elite hybrid Shanyou 63, which was the cultivar grown over the largest planting area in China during the 1990s. Populations derived from Shanyou 63 (F_2, RILs) were systematically used for yield trait QTL mapping in different environments, including both different locations and years. Yu et al. [2] used the $F_{2:3}$ population of 250 families and collected phenotypic data of yield and its three components including grains per panicle, 1,000-grain weight, and tillers per plant from replicated experiments of the $F_{2:3}$ population in 2 years. Whereas a total of 32 QTLs were detected for these four traits, only 12 QTLs were commonly detected in 2 years. Making use of the ratooning habit of rice, Li et al. [90] used vegetatively replicated ratooned plants from the 250 F_2 plants in the study of Yu et al. [2] to detect QTLs. A total of 20 distinct QTLs were detected for four yield traits; most of the QTLs detected in the ratooned F_2 population were also detected in the $F_{2:3}$ population [2]. Xing et al. [4] analyzed the main effects, epistatic effects, and their environmental interaction of QTL using 240 RILs derived from the same cross. A total of 29 QTLs were shown to have major effects on yield and yield-component traits, and 35 additive by additive interactions, which involved 58 loci, were also detected in this population. Their results indicated that both main effect QTLs and interactions play important roles in the genetic basis of yield and yield-component traits. In addition, two major QTLs for grain length on chromosome 3 and for grain width on chromosome 5 were commonly detected in the F_2 and RIL populations, which were later cloned [87, 91–93].

Inter-subspecies populations are frequently characterized by a large range of phenotypic variation, which is helpful to identify QTL. Xiao et al. [47] took a RIL population from a cross between *indica* 9024 and *japonica* LH422 to map QTLs for 13 traits, which included grain yield. Li et al. [48] used the F_2 and F_2-derived F_4 lines from a cross between *japonica* Lemont and *indica* Teqing to dissect the genetic basis for grain yield components. A total of 19 QTLs were identified, including 9 QTLs for 1,000-grain weight, 6 QTLs for grain number per panicle, and four QTLs for grain weight per panicle. Using a DH population derived from a cross between *indica* Zhaiyeqing and *japonica* Jinxi17, Lu et al. [94] detected a total of 22 QTLs for six traits including spikelets per panicle, grains per panicle, and 1,000-grain weight in at least one environment. Only 7 of these 22 traits were commonly detected in three environments. Several QTLs that determine the numbers of spikelets and grains per panicle were common across several environments. A high-resolution linkage map that was developed

through sequencing-based genotyping was useful for mapping QTL. Wang et al. [39, 95] developed a genetic map with high resolution by re-sequencing a RILs population that was derived from a cross between *indica* 93-11 and *japonica* Nipponbare. A total of 49 QTLs were identified for 14 traits, and some major QTLs with large effects were mapped to a small region.

Cultivar rice is domesticated from its wild ancestor, *Oryza rufipogon*. The considerable genetic diversity in wild rice benefits rice breeding by enabling identification of favorable genes from the wild rice through QTL mapping with the populations generated from crosses between cultivars and wild rice. Moncada et al. [9] used a *japonica* Caiapo widely planted in Brazil and an accession of *Oryza rufipogon* from Malaysia to construct a BC_2F_2 population in order to determine whether the trait-enhancing QTLs come from *O. rufipogon*. They identified 2 QTLs for yield and 13 QTLs for yield components under drought-prone and acidic soil environments, which Caiapo was adapted to thrive in. Alleles from *O. rufipogon* for more than half of the QTLs had a trait-enhancing effect. Septiningsih et al. [96] identified 42 QTLs for agronomic traits using a BC_2F_2 population from a cross between IR64 and *O. rufipogon*. One-third of alleles originating from *O. rufipogon* improved target traits.

The number of detected QTLs depends on the genetic diversity of parents of the mapping populations regardless of whether they are inter-subspecies, intra-subspecies or wild-cultivar-derived populations. Grain weight has the most QTLs that cumulatively explain most part of trait variation. This is in agreement with its high heritability. Only a small percentage of QTLs related to yield traits could be repeatedly detected across environments (Table 4.3). In the cross between Zhenshan 97- and Minghui 63-derived F_2 and RIL populations, among all the detected QTLs for yield traits, only one QTL on chromosome 1 and two QTLs on chromosome 5 make more than a 10 % contribution to phenotypic variation [2, 4]. In the 9024/LH422-derived RILs and BC populations, no QTLs were shown to have a phenotypic variation that exceeds 10 %. In the Lemont/Teqing-derived RILs population, one and two QTLs were identified with a contribution to phenotypic variation greater than 10 % in the ZAU and CNRRI experiments [97], respectively. However, none was detected with a contribution to phenotypic variation larger than 10 % in the F_2 population [98]. In the Caiapo/*O. rufipogon* BC population, two QTLs had individual contributions to phenotypic variation that exceeded 10 % [9]. In summary, few major QTLs for yield traits could be detected in primary populations.

5.2 QTL Clusters

Clusters of yield-related QTLs are frequently identified in individual populations (Fig. 4.2). However, it is difficult to determine whether linked QTLs share the same intervals or single QTLs have pleiotropic effects on related traits in primary mapping populations. A cluster of yield-related QTLs on the short arm of chromosome 9 was fine mapped to a 37.4-kb region that includes seven QTLs for grain weight, heading date, plant height, spikelets per panicle, grains per panicle, panicle length, and panicle density that were co-localized in this region [99]. Using a population from the cross between *Oryza rufipogon* and *Oryza sativa*, a QTL cluster that affects a range of aspects of plant architecture (including tiller angle, tiller number, leaf sheath length, leaf angle, culm length, and panicle number) was mapped to the short arm of chromosome 7 [100]. Detection of a QTL cluster that influences the eating quality of cooked rice on the short arm of chromosome 8 across three environments in a population of CSSLs [101] indicates that these QTLs display stable expression. In the population derived from Shanyou 63, a region that flanks *GS3* on chromosome 3 affects grain weight, grain length, and the ratio of grain length to width. Cloning of *GS3* confirmed that this region harbors a QTL with a major effect on grain length and minor effects on grain width and grain weight [87]. The *Ghd7*-flanking region on chromosome 7 affects grain yield, heading date, grain yield, and flag-leaf area. Accordingly, cloning of *Ghd7* clearly showed that variation of these target traits was caused by the pleiotropic QTL *Ghd7* [84, 85]. Although *Gn1* is an effective

QTL for increasing grain number detected in primary population, later research involving a *Gn1-NIL-F2* population dissected *Gn1* into two loci, *Gn1a* and *Gn1b* [102]. The *Gn1a* locus was mapped to between R3192 and C12072S, whereas *Gn1b* was mapped to the outside region of *Gn1a*. Obviously, two closely linked QTLs control grain number. The florigen genes *Hd3a* [103] and *RFT1* [104], both of which act downstream in the photoperiod pathway, are located 11.5 kb apart on chromosome 6. Hence, QTL cluster is the result of linked QTLs or a QTL with pleiotropic effects on multiple traits in a small region.

6 QTL Mapping by Association Analysis

Association mapping has three advantages over linkage analysis. Firstly, association mapping uses the widely available natural varieties rather than requiring the generated dedicated population for QTL mapping. Secondly, several alleles of each locus are captured once. Finally, the resolution of mapping to QTLs is much higher, even for a single gene. Association mapping requires extensive information about SNPs within the genome of the organism of interest. Thus, only species well-studied or well-annotated genomes are suitable for association mapping.

Owing to quick linkage disequilibrium (LD) decay, association mapping has been most widely applied to the study of human disease, specifically in the form of a genome-wide association studies (GWAS) [105, 106]. Then, GWAS was performed by scanning an entire genome for SNPs associated with a particular trait of interest in *Arabidopsis* and maize [107–110]. The resolution of association mapping is confirmed to be very high due to its rapid LD decay, which is approximately 1 kb for landraces of maize and 2 kb for diverse maize inbred lines. Very high-quality reference genome sequences are available for the *indica* and *japonica* cultivars of rice. Meanwhile, rice has rich resources of germplasm that include the two major subgroups *indica* and *japonica*, which provide good opportunities to conduct GWAS in rice. Huang et al. [40] sequenced 517 Chinese rice landraces and constructed a high-density haplotype map of more than 3.6 million SNPs. The respective genome-wide LD decay rates of *indica* and *japonica* were estimated at 123 and 167 kb, with wide variation across their genomes. Strong population structure was observed when GWAS was performed using 373 lines for 14 agronomic traits. A total of 37 association signals were found with a high threshold of P from the compressed mixed linear model. Of these, six peak signals were tied closely to previously identified genes, with a high resolution of less than 26 kb. Under more restricted conditions to detect associations, 80 associations were detected for the 14 traits. Huang et al. [41] made further GWAS with more than 900 accessions including 330 worldwide rice accessions, 100 Chinese accessions, and above-mentioned 517 landraces. Accordingly, a strong population structure still existed. They found 44 association signals, 32 of which were new. Zhao et al. [111] performed GWAS for 413 diverse accessions based on genotyping of 44,100 SNPs. A deep population structure was also observed. Whereas LD decay rates were 100–200 kb in *indica* rice, values of 300–500 kb were reported for *japonica*. Dozens of associations were detected for 34 traits. The fact that GWAS of each subpopulation independently generated different signal peaks indicated a rich genetic architecture of complex traits in *Oryza sativa*.

Compared with the previous linkage analyses, only a small proportion of cloned QTLs was identified in the three GWAS studies. Heading date is a complex trait that has been subject to both artificial selection and natural selection [112]. Taking heading date as an example, nine heading date QTLs have been cloned by positional cloning strategies. Nonetheless, no major heading date QTLs were identified by GWAS in 373 *indica* lines [41], and only five major heading date QTLs were tracked in 950 different rice varieties [41]. Coincidently, Zhao et al. [111] only detected one photoperiod-sensitivity heading date gene in their analysis of 413 lines. In addition, on average, a few associations were detected for each trait in the above-mentioned

three studies. This can be explained, at least in part, by the use of a strict threshold value to claim association. However, the main reason probably relates to deep population structure effects, which are not well controlled by statistical methods.

There is no doubt that GWAS can improve the power of QTL mapping and improve the resolution in rice. The interpretation of association signals will aid the identification of causal genes through GWAS, and GWAS will play an important role in QTL mapping in the future. However, owing to both the low rate of LD decay and deep population structure in rice, most minor genes cannot be mapped, and very few major QTLs can be narrowed down to single candidates. In other words, identifying the loci associated with complex traits by GWAS is challenging in rice, and linkage analysis is likely to remain necessary at least for the near future. As mentioned earlier, coupling of the potential of novel MAGIC populations with the advantages of LA and LD mapping should considerably facilitate QTL detection.

7 Map-Based Gene Cloning

In advanced breeding populations, such as NILs, target traits show the segregation characteristics of single Mendelian factors owing to the almost identical genetic backgrounds of individuals. To date, all successful QTL cloning studies have relied on the generation of advanced populations, indicating the likelihood that advanced populations are probably necessary for QTL cloning. Many genes affecting rice plant architecture, grain yield, and heading date have been isolated by map-based cloning. All of these are distributed across 11 chromosomes (Fig. 4.2, Table 4.4). Most of the genes control single traits, although two genes have pleiotropic effects on distinct traits.

The major QTLs *Ghd7* [85] and *Ghd8* [113] each control grain number per panicle, plant height, and heading date. Naturally occurring alleles of *Ghd7* showed a clear geographic pattern. Varieties that carry strong functional alleles of *Ghd7-1* and *Ghd7-3* are grown in tropical and subtropical regions in which there is a long and warm growing season. These two strong functional alleles allow rice plants to fully use the light and temperature resources by delaying the heading date to produce large panicles and more grain yield. However, varieties that carry *Ghd7-0* and *Ghd7-0a*, which are two nonfunctional alleles, are grown as early rice in two-rice crop systems in central and southern China and also in the high north latitude region in which the growth duration for rice is short. There are also several naturally occurring alleles of *Ghd8*. Strong functional allele has large effects on the three traits and vice versa. The mechanism that underlies these pleiotropic effects is not yet clear.

Pleiotropic gene probably regulates several enzymes involved in different metabolic pathways, which results in variations of distinct traits. A problem arises when selection of one trait favors one specific allele, while selection of a different trait favors another allele. Linkage drag can be broken by recombination. However, pleiotropic effects that conflict with rice production cannot be resolved by recombination. Searching for alleles that cause positive comprehensive effects on rice production in nature is one compromise. Genetic engineering is a newer option to resolve the problem when the molecular mechanism underlying pleiotropic effects is clear.

8 Functional Markers

Many studies over the past 20 years have accumulated a considerable amount of QTL-related information about rice production, grain quality, and tolerance of biotic and abiotic stresses. For non-cloned genes, linked markers are tools for marker-assisted selection. However, for cloned genes, gene-derived markers as functional markers, which are derived from polymorphic sites within functionally characterized genes directly associated with phenotypic trait variation, are more efficient to track specific alleles without worrying about the recombination between markers and genes. Thus, functional markers are more reliable and efficient than anonymous genetic markers. Functional markers are designed to distinguish between two alleles (e.g., a wild-type

Table 4.4 Yield-related genes identified by map-based cloning in rice

Gene	Encoded product	Target traits	References
Hd1	CO	Flowering	Yano et al. [121]
Hd3a	FT-like	Flowering	Kojima et al. [103]
Hd6	αCK2	Flowering	Takahashi et al. [122]
Ehd1	B-type RR	Flowering	Doi et al. [123]
Ghd7	CCT domain protein	Flowering, plant height and grains per panicle	Xue et al. [85]
Ghd8	CCAAT box binding domain	Flowering, plant height and grains per panicle	Yan et al. [113]
			Wei et al. [124]
Ehd3	PHD	Flowering	Matsubara et al. [125]
RID1/Ehd2	Zinc finger	Flowering	Wu et al. [126]
			Matsubara et al. [127]
Se13	PΦB synthase	Flowering	Saito et al. [128]
Gn1a	OsCKX2	Grain number	Ashikari et al. [102]
LOG	Cytokinin-activating enzyme	Panicle size	Kurakawa et al. [129]
DEP1	PEBP	Panicle architecture and grain number	Huang et al. [130]
DEP3	Patatin-like PLA2	Panicle architecture and grain number	Qiao et al. [131]
GS3	Transmembrane protein	Grain shape and grain length	Fan et al. [87]
GW2	RING-type protein	Grain width	Song et al. [132]
GW5	Nuclear protein	Grain width	Weng et al. [93]
			Shomura et al. [92]
GS5	Putative serine carboxypeptidase	Grain width	Li et al. [133]
GIF1	Cell-wall invertase	Grain filling and grain weight	Wang et al. [134]
MOC1	GRAS family	Maintain tiller number	Li et al. [135]
Sd1	*Os20ox2*	Plant height	Spielmeyer et al. [136]
			Ashikari et al. [137]
			Monna et al. [138]
IPA1	*OsSPL14*	Ideal plant architecture	Jiao et al. [139]
TAC1	Unknown protein	Tiller angle	Yu et al. [140]
LAZY1	Grass-specific protein	Tiller angle	Li et al. [141]
RCN1	PEBP	Flowering and enlarge panicle	Nakagawa. [142]
RCN2	PEBP	Flowering and enlarge panicle	Nakagawa. [142]
FLO2	Novel protein	Grain size and starch quality	She et al. [143]

allele and a mutated allele), which result in distinct loss-of-function or gain-of-function phenotypes. A list of functional markers is provided in Table 4.5. Functional markers can identify two different alleles that generate different phenotypes. For example, functional markers of *Badh2* can easily distinguish non-fragrant from fragrant rice and differentiate between two kinds of fragrant rice [114]. Yang et al. [115] took advantage of the 136-bp deletion of a wide compatibility gene (*S5-n*) and designed a functional InDel marker S5136. Yang et al. [116] designed an InDel marker RID14, which is a valuable functional marker for identification of weedy rice with a red pericarp [117].

For the purposes of quantitative trait improvement, it would normally be more efficient to mine alleles with weak or strong functional alleles rather than nonfunctional alleles. For instance, the pleiotropic gene, *Ghd7*, has five haplotypes or functional alleles with a distinct geographic distribution pattern. Naturally occurring *Ghd7* favorable alleles are specific to ecotypes and genetic backgrounds adapted to particular cropping seasons [85]. Three functional markers (SF28, RGS1, and RGS2) have

Table 4.5 Functional markers of important yield-related genes in rice

Genes	Target traits	Causal mutation	Functional markers	References
Badh2	Rice fragrance	8-bp deletion	FMbadh2-E7	Shi et al. [114]
		7-bp deletion	FMbadh2-E2A	
			FMbadh2-E2B	
S5-n	Hybrid sterility	136-bp deletion	S5136	Yang et al. [115]
rc	Red pericarp	14-bp deletion	RID14	Sweeney et al. [117]
				Yang et al. [115]
GS3	Grain size	C-A mutation	SF28	Fan et al. [42]
		(AT)n repeat	RGS1	Wang et al. [118]
		(TCC)n repeat	RGS2	

been derived from *GS3* [42, 118]. Whereas SF28 can identify long from medium and small grains, RGS1 and RGS2 can distinguish between medium and small grains.

Obviously, functional markers can enable more refined selection by capturing more varied functional alleles than markers linked to target genes. Currently, about 600 genes involved in rice yield, quality, disease, and insect resistance have been cloned [119]. Association analysis of these genes should reveal naturally occurring alleles. Functional markers that enable us to distinguish between varied alleles could be used to design a multipotent diagnostic array for rice genetic improvement. One such array can be used to simultaneously genotype hundreds of genes controlling several traits, enabling greatly enhanced selection efficiency.

9 Prospects

Advances in genomics and functional genomics have considerably promoted progress in rice genetics. Whatever mapping method is used for mapping QTLs, the current bottlenecks in QTL resolution are the collection of high-quality phenotypic data and a reasonably large population size, and not the density of the available markers. Establishment of a phenotyping platform that will enable reliable collection of data is becoming more and more important [120]. Notwithstanding the recent surge in the popularity of using GWAS to detect plant QTLs and genes, GWAS is probably unable to reveal its full potential in the mapping of rice QTLs owing to the slow rate of LD decay (150–250 kb on average) and its complex population structure. Hence, a linkage analysis strategy is still very important for QTL mapping and cloning in the near future. The MAGIC approach combines the advantages of linkage analysis and LD and avoids their disadvantages. Thus, developing such a population will provide tremendous opportunities for fine mapping of QTLs and effective identification of agronomically important alleles. Many major QTLs or genes that regulate yield components, disease resistance, and insect pest resistance have been cloned or fine mapped. Functional markers derived from these genes provide convenience to complete selection at the seedling stage under normal growth conditions. However, most functional markers mainly developed by the casual mutation resulted in loss-of-function or important functional modification. However, for most quantitative traits, varied functional alleles would be more attractive for use in breeding. These alleles would produce diverse functional markers as indicators of special phenotypes. Haplotype-based selection with functional markers for multiple loci and genome selection for genetic background will greatly accelerate the rice breeding process.

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Transposable Element Dynamics in Rice and Its Wild Relatives

5

Ning Jiang and Olivier Panaud

1 Introduction

Transposable elements (TEs) were first discovered by Barbara McClintock in the 1940s using maize (*Zea mays*) as a model organism [1]. Based on their transposition mechanism, TEs fall into two classes. Class I, the retrotransposons, use a "copy and paste" mechanism and utilize an RNA intermediate for transposition [2]. Class I elements can be further divided into several groups, including the long terminal repeat (LTR) elements, long interspersed nuclear elements (LINEs), and short interspersed nuclear elements (SINEs). During their transposition, the element mRNAs are converted into cDNA through the action of reverse transcriptase which is encoded within the element, and the TE cDNAs are then inserted into a target site in the genome. Due to their replicative transposition mechanism, class I elements can amplify very rapidly and contribute the largest portion of most plant genomes in terms of DNA content. Class II, the DNA transposons, are often associated with terminal inverted repeats (TIRs) and transpose via a DNA intermediate [3]. In general, DNA transposons excise from one site and reinsert elsewhere in the genome, resembling a "cut and paste" mechanism. In plants, there are several superfamilies of DNA transposons including *Ac/Ds* (*hAT*), *Spm/dSpm* (CACTA), *Mutator/Mutator*-like element (MULE), *Tc1/Mariner/Stowaway*, *PIF/Harbinger/Tourist*, and *Helitron*. When an element inserts into a genomic site, a small piece flanking sequence in the insertion site is duplicated and this sequence is called target site duplication (TSD). Each superfamily creates a unique length of TSD that can be used to identify the element. *Helitrons* are an exception to this classification because they do not generate a TSD [4].

Based on their coding capacity, both classes of TEs can be divided into autonomous and nonautonomous elements. Autonomous elements encode the protein products (Tpase or GAG-POL) required for their transposition. Nonautonomous elements do not encode the relevant products and rely on their cognate autonomous elements for transposition. When the nonautonomous *Tourist* and *Stowaway* elements were initially identified from plants, it was not clear what type of autonomous elements were associated with these elements [5, 6]. Hence, it was impossible to assign them to a specific family. As a result, they were classified as "miniature inverted repeat transposable elements" (MITEs) based on their structural features. About a decade later, it was revealed that *Tourist* elements are related to *PIF/Harbinger*

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elements [7–9], and the *Stowaway* elements are related to *Tc1/Mariner-like* elements (MLE) [10–12]. Technically MITEs can refer to any small TEs (<500 bp) with TIRs, although, it is the term most commonly used for *Tourist* and *Stowaway* elements due to the historical reasons.

Although rice (*Oryza sativa*) is a close relative of maize, the history of TE study in rice is distinct from that in maize in many aspects. On one hand, TE study in rice was not initialized until the early 1990s, which is half a century after TE was first discovered in maize. On the other hand, the discovery of TEs in maize was triggered by mutation phenotypes created by novel insertions of TEs, which represents a forward genetics approach. In contrast, the majority of TEs in rice were mined from genomic sequences, followed by characterization at different levels, which resembles a reverse genetics approach. Beside these differences, TE studies in maize and rice do share features: one of these is the importance of the *waxy* gene in the initial characterization of TEs in these two grass species.

Waxy encodes a granule-bound starch synthase which determines the amylose content in developing grains and pollen, an important agronomic trait. This gene is present in single copy in both maize and rice and is dispensable for plant growth. For this reason, the *waxy* locus in maize harbors many mutations and most of them are caused by TE insertions. Those elements include *Ac*/*Ds*, *En*/*Spm*, *B2* (the first *Tourist* element), and *G* retrotransposons [5, 13–16], which greatly facilitated the characterization of TEs in maize. In fact, the initial *Ds* element identified by McClintock was located adjacent to the *waxy* locus in maize [1]. In the early 1990s, the rice *waxy* gene was cloned using the maize *waxy* gene sequence as a probe [17]. The comparison of waxy gene sequence between Asian rice (*O. sativa*) and Africa rice (*O. glaberrima*) as well as that in maize led to the discovery of the first transposable elements in rice [18]. One element, called p-SINE1 (plant SINE #1), is a SINE located in intron 10 of the rice *waxy* gene. The other element, called *Tnr1* (transposable element in rice #1), containing TIRs, is located in intron 13 of this gene [18]. Subsequently, it was shown that *Tnr1* is a *Stowaway* element [6].

Despite its brief history, the study of TE in rice achieved significant progress in a relatively short period. These include the activation of LTR elements in tissue culture, the discovery of the first active MITE in any organism, the development of novel genomic strategy for systematic identification of active elements, the large-scale gene duplication by Pack-MULEs and retrotransposons, the contribution of LTR elements to the variation of genome sizes, the horizontal transfer of TEs among genomes, and the consequence of fast amplification of TEs on genome and genes. Such a burst of new knowledge about TEs has not been reproduced in any other plant. In this chapter, we focus on the unique insights inferred from TE studies in rice.

2 Composition of Rice TEs

Rice has a relatively small genome (430 Mb) compared to other grasses. Even if so, repetitive sequences comprise nearly half of the genome and most of them are TEs (i), including both class I and class II elements. Among the five known groups of class I elements [19], three, i.e., the LTR retrotransposons, LINEs, and SINEs have been found in the rice genome [20]. However, LTR retrotransposons outnumber by far the other two orders, a common feature of most plant genomes. Consequently, most studies published so far on rice class I elements focus on LTR retrotransposons because of their significant impact on genome structure and evolution in this species in comparison with LINEs and SINEs. About 1/4 of the rice genome is comprised of LTR and non-LTR retrotransposons [21] (Table 5.1). The first reports on TE composition of the rice genome, including that of the complete genome sequence [20], did not mention about the number of LTR-retrotransposon families found in the Nipponbare cultivar, although this information is needed in order to fully understand the role of LTR retrotransposons in shaping the rice genome. In fact, the classification of TEs into distinct families is often not straightforward and made difficult mostly by the frequent occurrences of post-insertional recombination events that give

Table 5.1 Transposable elements and other repetitive sequences in rice genome (cv. Nipponbare)

Class	Superfamily	No. of families	Copy number[a] (×1,000)	Coverage (Mb)	Fraction of genome (%)
Class I	LTR/*Copia*	122	4.1	13.4	3.6
	LTR/*Gypsy*	160	11.5	57.9	15.5
	LTR/LARD/TRIM	58	5.1	14.3	3.8
	Total LTR	340	20.7	85.6	22.9
	LINE	88	3.9	5.6	1.5
	SINE	32	4.5	1.3	0.4
	Total non-LTR	120	8.4	6.9	1.9
	Total class I	460	29.1	92.5	24.8
Class II	CACTA	114	7.4	15.3	4.1
	hAT	228	11.1	5.8	1.6
	MLE/*Stowaway*	76	36.8	8.7	2.3
	MULE	417	32.0	20.5	5.5
	PIF/*Tourist*	223	37.6	12.0	3.2
	Helitron	246	10.1	12.5	3.3
	DNA/unknown	13	2.6	1.6	0.4
	Total class II	1,317	137.6	76.4	20.4
Total transposable elements		1,777	166.5	168.9	45.2
Other repeats		402	6.5	6.2	1.7
Total repeats		2,179	173.0	175.1	46.9

[a]An element with both termini is considered as one copy; an element with one terminus is considered as 0.5 copy. Fragmented elements with no terminus are not considered

rise to chimeric forms, some of which can in turn be mobilized in trans and give rise to new insertions thus sharing homology with clearly distinct families. Chaparro et al. [22] tentatively established a curated database of the rice LTR retrotransposons, giving a particular attention to the identification of distinguishable families (www.retroryza.fr). This database contains 242 families that can be freely downloaded and offers other functionalities such as the annotation of any rice sequence for LTR retrotransposons. More recently, the same group developed a new and improved automated LTR-retrotransposon classification procedure, based on clustering (ElBaidouri et al. submitted). Conceptually, classification by clustering is more robust than classical methods that are based on the use of a consensus sequence combined with genome-wide homology searches. In particular, clustering allows the classification of large retrotransposon derivatives [23] into the same family as their autonomous counterpart. LARDs are nonautonomous elements, with LTRs that are similar to that of known LTR retrotransposons and an internal region that is not. So far in rice, four LARDs have been identified: *Dasheng* [24], nonaCRR [25], Spip, and Squiq [26] that share high sequence identity in their LTRs with the LTR-retrotransposons RIRE2, CRR, RIRE3, and RIRE8, respectively. The new classification procedure proposed by El Baidouri et al. allows unambiguous classification of these four LARDs together with their autonomous counterparts, while distinguishing them in two distinct subfamilies. A complete re-annotation of the Nipponbare genome following this new approach revealed that it harbors 340 distinct families of LTR retrotransposons, most of which being low or single copy. Of these, 42 families have more than ten copies in the genome which, taken together, represent 2,896 intact elements.

The rice genome harbors all types of plant DNA transposons (class II elements). Compared with retrotransposons, DNA transposons account for slightly less of the genomic fraction (Table 5.1). Nevertheless, the copy number of DNA transposons is about fourfold of that of retrotransposons. Among all sequenced plant

genomes, rice has the highest ratio of DNA/RNA elements in terms of copy number. This is mainly due to the presence of numerous small nonautonomous DNA transposons especially MITEs (*Stowaway* and *Tourist* elements), which are frequently associated with genes [27]. In addition to *Tourist* and *Stowaway* elements (as well as their corresponding autonomous elements), other DNA transposons also have amplified to a considerable degree in the rice genome. There are over 30,000 MULEs which has the highest contribution (5.5 %) to genome size among DNA elements. This is followed by CACTA elements, which contributes 4.1 % of the rice genome. *Helitron* and *hAT* elements are less abundant in genomic mass, yet each of them is still associated with over 10,000 family members (Table 5.1).

3 Active TEs in Rice

Since the initial discovery of TEs in rice was through genomic sequence mining instead of mutations caused by TE insertions, it is understandable that the identification of active elements occurred relatively late compared to that in other plants such as maize, tobacco, and snapdragon [1, 28, 29]. Nevertheless, the availability of rice genomic sequence and other genetic and genomic resources provided a unique advantage for the identification of transpositionally active TEs as well as understanding the mechanism and consequence of TE activation.

Although class I elements densely populate the rice genome, only ten transpositionally active families have been identified so far. These are the LTR-retrotransposons *Tos17* [30]; *Lullaby* [31]; BAJIE, Osr10, Osr37, RIRE2, RIRE3, RN363, and RN216 [32]; and the LINE *Karma* [33]. Altogether, the active copies of these ten families represent a very small proportion of the Nipponbare genome, which contrasts with the fact that the genome harbors several hundreds of thousands of TE-related sequences that make up 169 Mbp (Table 5.1). Most of the TEs found in complex eukaryotic genomes are not functional, either because they are the target of silencing processes that impede their proliferation in the genome [34] or because they have been structurally altered by mutations [26]. One of the major difficulties in identifying active TEs is the development of a suitable screening method for the detection of new insertions. The discovery of both *Tos17* and *Karma* elements was achieved through the cloning and sequencing of cDNAs that were amplified through PCR using primers designed in the conserved domains of the reverse transcriptase gene. *Tos17* has been shown to be activated during in vitro culture [30]. This finding has been exploited to generate large mutant collections that are now used by the rice community as reverse genetics tools for functional genomics studies [35]. Although this method (i.e., the use of PCR to amplify transcripts) is robust and straightforward, it may not be suitable for an exhaustive survey of all transcriptionally active LTR retrotransposons because of the bias associated with PCR amplification. The approach followed by Picault et al. [31] aimed to circumvent this limitation: the authors used a genomic microarray that represents the complete repertoire of the rice LTR retrotransposons (the transposome array) to conduct various transcriptomic surveys, including that of callus culture (known to activate the transposition of *Tos17*). This work led to the discovery of the second active LTR retrotransposon in rice (*Lullaby*). The recent work by Sabot et al. [32] was based on the use of next-generation sequencing (NGS) technologies. The authors sequenced a rice mutant line that was regenerated from callus in vitro culture. Using paired end mapping (PEM) of Illumina sequencing data, they detected several LTR-retrotransposon insertions that are not present in the reference sequence of Nipponbare genome, thus providing the first genome-wide transpositional landscape of the rice genome. The use of similar strategies (i.e., high-throughput sequencing combined with suitable bioinformatic analysis tools) on various physiological conditions (including both biotic and abiotic stress) may help to better understand the role played by LTR retrotransposons in the plant genomic response to environmental stimuli.

Seven years after the identification of the first active LTR element, the first active DNA element in rice was discovered independently by three

labs through two distinct approaches [36–38]. This element, called *mPing*, was the first active MITE found from any organism. One lab identified *mPing* through a mutation called *slender glume* (*slg*), caused by the insertion of this element [38], which resembles the classical pathway for TE discovery. A genomic/computational method was employed by the other two labs [36, 37]. With this method, candidate active MITEs were identified through computational search of available genomic sequence. The rationale for this search is that if an element is active, or was active in the recent past, identical or similar copies should be identified in the genomes. Among the 52 copies of *mPing* in Nipponbare, 40 are identical to each other, strongly suggesting a recent activity. Since *mPing* appeared to be a good candidate for an active MITE, its transposition activity was tested in tissue culture, where excisions and new insertions of *mPing* were observed [36, 37]. In the Nipponbare genome, there is a single copy element, called *Ping*, encoding a transposase similar to the previously described *PIF*/*Harbinger* elements. Based on sequence alignment, it is apparent that *mPing* is a deletion derivative of *Ping* [36], which is the putative autonomous element responsible for the mobilization of *mPing*.

Rice has two subspecies: *indica* and *japonica*. An intriguing fact is that *Ping* is only present in *japonica* cultivars such as Nipponbare and absent from *indica* cultivars. However, rapid increase of copy number of *mPing* in tissue culture was observed with C5924, an *indica* variety [36]. This prompted the search for the autonomous element providing the transposition machinery for *mPing* to move in *indica* cultivars. It turned out that in C5924, there are several copies of *Pong*, which is 70 % similar to *Ping* and *mPing* at the nucleotide level and also encode a *PIF*/*Harbinger* like transposase [36]. Subsequently, it was shown both *Ping* and *Pong* are capable of inducing transposition of *mPing* in Arabidopsis and yeast [39, 40]. The mobilization of a nonautonomous element by a distantly related autonomous element is called "cross-mobilization." In addition to its unusual transposition mechanism, the copy number of *mPing* varies dramatically among different rice varieties, ranging from 1 to 1,000 copies [41]. It was estimated that in certain rice cultivars *mPing* was increasing its copy number by ~40 copies per plant per generation [41]. Most of these newly amplified *mPing* elements insert near genes, which is consistent with the previous findings about MITEs. Surprisingly, most of the *mPing* insertions either upregulate or have no detectable impact on the expression of nearby genes [42]. This is in contrast to other reports showing a suppressive effect of TEs on the expression of adjacent genes [43].

Subsequent to the discovery of *mPing*, two nonautonomous DNA elements, *nDart* (DNA-based active rice transposon) and *dTok*, were reported to be active in rice plants. Both *nDart* and *dTok* belong to the *hAT* superfamily, and the termini of the two elements are similar to a certain degree (70 % similarity). As the founder elements (*Ac*/*Ds*) of this superfamily, both *nDart* and *dTok* elements were identified through the visible mutation phenotype they caused. The *nDart* element was first identified as an insertion into a Mg-protoporphyrin methyltransferase gene, which caused an albino phenotype [44]. A second *nDart*-related mutant, which resulted from a spontaneous mutation that led to leaf variegation, is caused by an *nDart* insertion that interrupted the function of a chloroplast protease gene, *OsClpP5* [45]. The *dTok*-related mutant was a naturally occurring mutant with multiple pistils in a single flower (a normal rice flower only contains one pistil) due to a *dTok* insertion into the *FON1* (flower organ number1) gene [46]. Interestingly, the *dTok* element is only active in reproductive organs, not in vegetative tissues.

The PEM method mentioned above is not only effective in identifying active retrotransposons but also useful for the discovery of active DNA elements [32]. With this method, four novel active DNA TEs were discovered [32]. The first element belongs to the *hAT* family but is distinct from *nDart* and *dTok* despite the fact that they have approximately the same size (Table 5.2). The remainder of the elements is small nonautonomous MULEs, which are the first active MULEs from rice. Recently, a putative autonomous MULE was reported to be active in the progeny plants derived from rice tissue culture [47].

Table 5.2 Active DNA TEs in rice

Element	Superfamily	Element size (bp)	Copy number	Tissues with transposition activity	References
mPing	*PIF/Harbinger/Tourist*	419–450	52	Tissue culture from mature embryos	[36]
				Anther culture	[37]
				Plants from seeds after γ-ray irradiation	[38]
				Rice plants with DNA introgression from *Zizania latifolia*	[51]
				Plants from seeds subject to hydrostatic pressure	[54]
				Somaclonal mutants (derived from tissue culture)	[48]
				Plants from seeds in spaceship for 18 days	[55]
				Tissue culture from mature embryos	[49]
				Plants derived from an incompatible cross-pollination	[53]
				Anther culture	[50]
				Geminating seeds after treatment with topoisomerase II inhibitor	[56]
Ping	*PIF/Harbinger*	5,353	1	Anther culture	[37]
Pong	*PIF/Harbinger*	5,166	5	Tissue culture from mature embryos	[36]
				Rice plants with DNA introgression from *Zizania latifolia*	[51]
				Plants from seeds in spaceship for 18 days	[55]
nDart	*hAT*	588–690	18	Intact plants derived from a cross between two japonica cultivars	[44]
				Intact plants from seeds treated by 5-azaC	[45]
				Rice plants with DNA introgression from *Zizania latifolia*	[52]
dTok	*hAT*	631–668	25	Reproductive organs of plants	[46]
Mite#1	*hAT*	686	1,696	Tissue culture	[32], this study
Mite#2	MULE	380	2,554		
Mite#3	MULE	376	59		
Tami2	MULE	256	42		
mGing/Gaijin	*PIF/Harbinger/Tourist*	140	1,000	Anther culture	[50]
Os3378	MULE	4,395	4	Somaclonal mutants (derived from tissue culture)	[47]

In contrast to maize, where many active TEs are identified through the spontaneous mutations that they created in plants, most active TEs in rice are discovered through certain activation processes (Table 5.2). The most ubiquitous procedure to activate TEs in rice is through tissue culture [30, 32, 36, 37, 47, 48, 50], followed by hybridization or DNA introgression [44, 51–53]. Abiotic stresses including isotope irradiation [38], hydrostatic pressure [54], being space flown [55], and application of certain chemicals [45, 56] are also responsible for activation of elements. In many cases, the activation of TEs is accompanied by a reduced degree of DNA methylation [49, 57, 58]. From this point of view, it is not surprising that the overexpression of rice DNG701 gene, which is responsible for reducing the methylation level, led to the transposition of *Tos17* in the transgenic lines [59].

4 Target Selection of TEs and Centromere-Specific Elements

Although TEs appear to insert throughout the genome, many elements demonstrate a certain degree of target site preference [60]. TEs make their target selection at different levels. Some

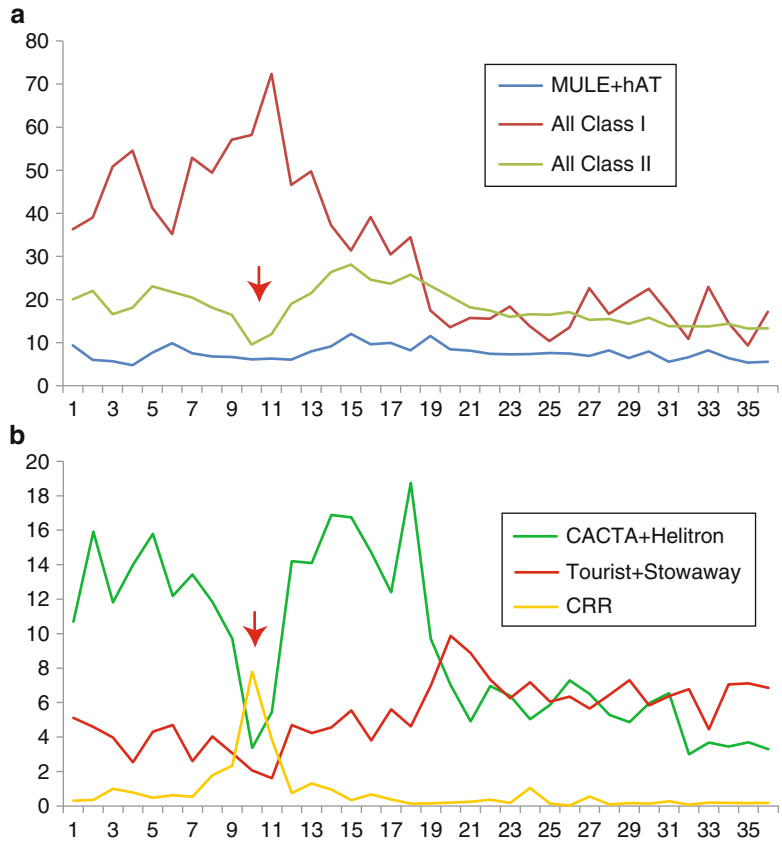

Fig. 5.1 The distribution of different types of TEs on chromosome 4. *X*-axis indicates the physical distance (Mb) on the chromosome and *Y*-axis stands for the genome fraction (%) each type of TE represents. The bin size is 1 Mb. The *red arrow* points to the position of centromere

elements choose to insert into specific sequences, which is called "sequence specificity." Such specificity can be revealed by the TSD of the element. In rice, most *Tourist* elements create a trinucleotide TSD "TNA" [9], and *Stowaway* elements always create a dinucleotide TSD "TA" [6, 12]. *Helitron* does not create a TSD, yet it always inserts into "AT" dinucleotide [4]. The target site for class I elements is generally not sequence specific; however, some of them demonstrate certain biases when they insert into the genome. For example, *Dasheng* and its putative autonomous element, RIRE2, have a strong bias for A or T in a few positions inside or flanking the TSD, whereas such bias is not observed with another LTR retrotransposon, RIRE3 [61].

In addition to sequence specificity, different TEs have distinct niches on the chromosome. Figure 5.1 demonstrates the distribution of different TEs on rice chromosome 4. This chromosome is chosen due to the dramatic structural difference between its short arm and long arm. The short arm of chromosome 4 is highly heterochromatic and contains few genes [62], whereas most of the long arm is euchromatic, with high gene density. In general, the distribution of class I and class II elements is complementary (Fig. 5.1a), with class I elements in heterochromatic regions and class II elements in euchromatic regions. However, different families of DNA elements vary in their distribution patterns. *hAT* and MULEs are largely evenly distributed along the chromosome (Fig. 5.1a). *Tourist* and *Stowaway* elements are significantly enriched in the long arm, which is consistent with the notion that they are frequently associated with genes (Fig. 5.1b). The most dramatic distribution pattern was observed for CACTA elements and *Helitrons*, with a bimodal

distribution that distinguishes them from any other elements. They are enriched in the short arm as well as the region between the centromere and the long arm and are almost absent from the long arm and the centromeric region (Fig. 5.1b). The variation in distribution patterns displayed by different elements suggests that the relevant transposition machinery interacts with different chromatin components or that these elements are subject to different selection pressures.

Centromeres are the chromosomal domains essential for the assembly of the chromosomal structures that mediate faithful segregation at mitosis and meiosis. Centromeres interact with the spindle apparatus to enable chromosome disjunction. Like other chromosomal domains, the centromere/kinetochore complex is composed of DNA and structural proteins. One of the centromere-specific proteins is called CENH3, which replaces the regular histone H3 in centromeric chromatin and is critical in the establishment of kinetochores in various organisms. In plants, satellite DNA (centromeric repeats) and LTR retrotransposons are the main DNA components in centromeres [63]. As mentioned above, most LTR elements are concentrated in centromeric/pericentromeric regions (Fig. 5.1), yet they are also found in other chromosomal domains. Nevertheless, there is a special group of LTR elements that are almost exclusively located in centromeric regions (Fig. 5.1b), and they are called "centromeric retrotransposon (CR)" (CRR in rice and CRM in maize). Unlike other retrotransposons that diverge rapidly, the CR elements are conserved and specifically located in centromeres in almost all the grass species [63].

Retrotransposon fragments and promoters drive centromere satellite DNA expression [64]. Without such expression, condensation of the centromere would be lost. The CRM retrotransposon directly interacts with CENH3 [65], and the interaction is sufficient to induce centromere identity and function. It was speculated that the centromere-associated transcripts, including those from CR elements, might be essential in the recruitment of CENH3 [63]. Therefore, the CR elements and their transcripts may facilitate the chromosome segregation through their interaction with CENH3. Interestingly, the recently active CRM elements are found in the center of centromeres and relatively ancient CRM elements are located in pericentromeric regions. This suggests that the centromere is a dynamic complex which continuously recruits newly transposed elements in the center of the centromere and "pushes" older elements to the flanking regions [66]. Nevertheless, the role of CR elements does not seem to be indispensable. In *Oryza brachyantha*, a wild relative of rice, the CRR element is absent from the centromeres [48]. Instead, the centromeres are occupied by a distinct LTR element, called FRetro3, that plays an equivalent role in regard of interacting with CENH3. Moreover, recent studies suggest that new centromeres (or neocentromeres) are devoid of TEs [67, 68]. This may imply that occupation of centromeres by TE occurs after the establishment of initial centromere activity, which enhances the stability of the centromere through interaction with centromere-specific histone variants [67]. The replacement of centromeric TEs between related species suggests that TEs in centromeres may play a key role in reproductive isolation and the emergence of new species.

5 The Contribution of LTR Elements to the Genome Size Variation in *Oryza*

One of the most striking features of LTR retrotransposons is their impact on genome size variation in plants. Because of their "copy and paste" mode of transposition, active families can generate a high number of copies that may significantly modify the size of their host genome. It is now clearly established that genome size variation in Poaceae is caused by differential retrotranspositional activity, besides polyploidy [69–71], and that there is a strong correlation between genome size and LTR-retrotransposon content in most evolutionary lineages. Interestingly, the work of Piegu et al. [72] and Ammiraju et al. [73] provided the first evidence of the dynamics of the process through which the amplification of retrotransposition leads to genome size variation in plants. Both studies concerned the genus *Oryza*. The

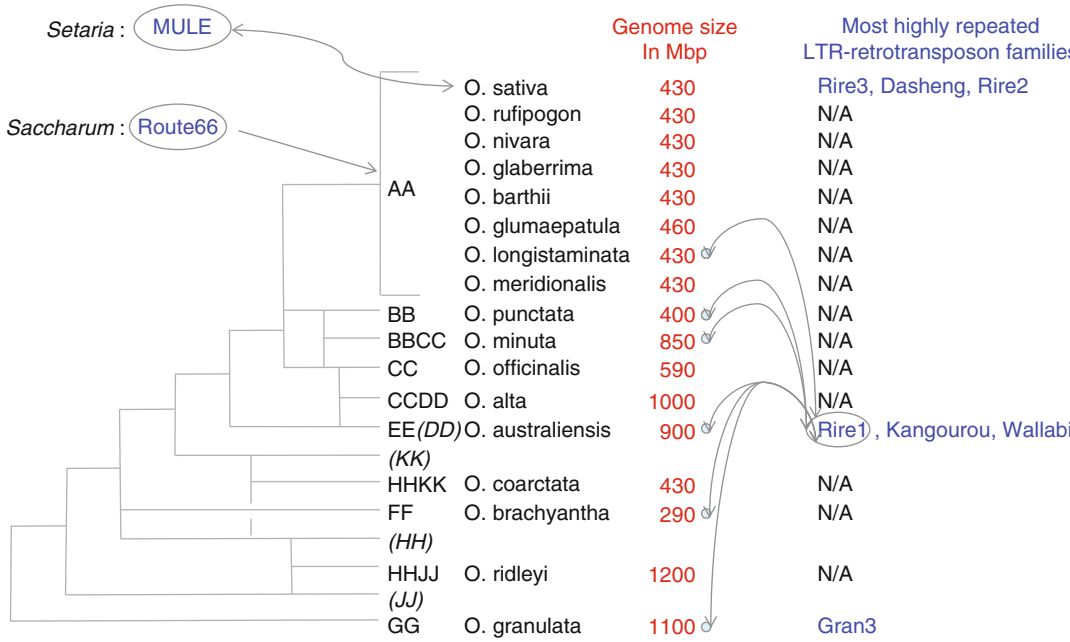

Fig. 5.2 Phylogenetic tree of *Oryza* genus with an emphasis on genome size and TEs. The topology of the tree is derived from previously published work of Ge et al. [89]. *Dotted arrows* correspond to horizontal transfers that have been evidenced in three distinct publications. Diao et al. [93] for the HT of a MULE between Setaria and rice; Roulin et al. [95] for the transfer of the retrotransposon route66 between Saccharum and rice, and Roulin et al. [94] for the multiple horizontal transfer of RIRE1 within the *Oryza* genus

authors showed that the large size of the wild species *O. australiensis* [72] and *O. granulata* [73] genomes, compared to that of the cultivated species *O. sativa*, can be solely explained by the activity of a few LTR-retrotransposon families. For instance, half of the genome of *O. australiensis* is composed of 100,000 copies of LTR retrotransposons originating from only three families (*RIRE1*, *Kangourou,* and *Wallaby*) (Fig. 5.2). Moreover, this dramatic change in genome size occurred through very recent bursts of retrotransposition, i.e., within the last 2 million years, which is posterior to the speciation in both cases. Following such massive genomic amplifications, a strong bias of mutations towards deletions led to the quick elimination of LTR-retrotransposon-related sequences. This has been first evidenced by Ma and Bennetzen [74] through an elegant comparative genomic survey of LTR-retrotransposon insertions between the two closely related cultivated rice species *O. sativa* and *O. glaberrima* which showed that the half-life of LTR-retrotransposon-related sequence is ~2 My. Vitte et al. [26] also examined the TE elimination process in rice through a detailed analysis of several LTR-retrotransposon families and concluded that small deletions cannot solely account for the rapid elimination of LTR retrotransposons, thus suggesting that additional forces, such as selection, may be involved. More recently, Tian et al. [75] showed that the elimination rate of TEs through recombination depends on the recombination rate as well as the gene density of the genomic regions where they insert, therefore suggesting that the accumulation of LTR retrotransposons in certain regions of the genome (e.g., the pericentromeric regions) may be the result of both the retrotransposition bursts and a lower elimination rate through recombination. In any case, taking the genome as a whole, all these studies have clearly shown that plant genomes undergo a fast genomic turnover caused by the combined effect of the two

counteractive forces: the retrotransposition and the deletion of TE-related sequences (mostly through recombination).

6 Gene Duplication and Acquisition by TEs in Rice

The first incidence of gene acquisition/duplication by TEs in plants was reported more than 20 years ago, when the *Mu1*/*Mu1.7* elements in maize were shown to contain part of a gene of unknown function called *MRS-A* [76]. Nevertheless, the scale of gene acquisition by this type of element, or by DNA transposons in general, was not realized until the release of the complete genome sequence of rice, where thousands of gene-carrying MULEs (called Pack-MULEs or transduplicates) were found [77, 78]. As a result, duplication, amplification, and mobilization of genes and gene fragments by Pack-MULEs represent one of the major mechanisms for shuffling of coding and regulatory sequences in rice. Several models were proposed to explain the formation of Pack-MULEs [76, 79], yet none of them has been verified so far. Recent studies indicate that Pack-MULEs preferentially acquire GC-rich regions that are associated with open chromatin, suggesting that accessibility of the DNA sequences may determine the acquisition preference [80].

The acquisition of genomic sequences by retrotransposons likely occurs during the transposition process. One component of the retrotransposition machinery of an LTR element is the virus particle in which the element mRNAs are included and subsequently converted into cDNAs [81]. During this process, mRNAs of normal genes can be fortuitously packed into the particle and thereafter retrotransposed. Since these retrogenes are generated through reverse transcription and transposition, they are distinguished from their parental forms by the presence of a polyA tract, the lack of introns and promoters, and the presence of a TSD. Some of the retrogenes are associated with the relevant elements, i.e., gene fragments may be found between the two LTRs of a single LTR element. Other retrogenes may be present independently, making it difficult to deduce the element responsible for their formation. In rice, over 1,000 genes duplicated through retrotransposition (retrogenes) have been identified, and many recruited new exons from flanking regions, resulting in the formation of chimeric genes [82].

Given the abundance of gene-carrying elements in rice, a critical question is whether some of them play any functional role. Emerging evidence suggests this is likely the case. A recent study indicates most retrogenes are expressed so they may have acquired new functions [83], which is confirmed by the constraint of sequence substitutions. For a nucleotide sequence, nonsynonymous substitutions refer to mutations that lead to the alteration of amino acids, and synonymous substitutions refer to mutations that do not change amino acid sequence. The ratio of nonsynonymous to synonymous substitution rates should be smaller than one under purifying selection [84]. For both Pack-MULEs and retrogenes, it has been shown that selection pressure is significant between the acquired regions in TEs and their parental genes, despite the fact that most acquired regions only represent gene fragments [82, 85]. In addition, most Pack-MULEs generate small RNAs and share small RNAs with their parental genes, which are correlated with a reduced expression level of these genes. This suggests that after acquisition, the acquired and amplified fragments may negatively regulate the expression of their parental genes, through the formation of small RNAs [85]. Finally, Pack-MULEs frequently insert into 5′ of genes, fuse with the downstream gene, and modify the structure and the GC content of the relevant gene [80] (Fig. 5.3). These data suggest that gene-carrying elements are influencing genes and genome structure through a variety of mechanisms in rice.

7 Paleontology of LTR Elements and Phylogenic Relationships of Their Host Species

As mentioned above, one particular feature of the transposition cycle of class I elements is their “copy and paste” mode of transposition. The evolutionary fate of a newly inserted LTR

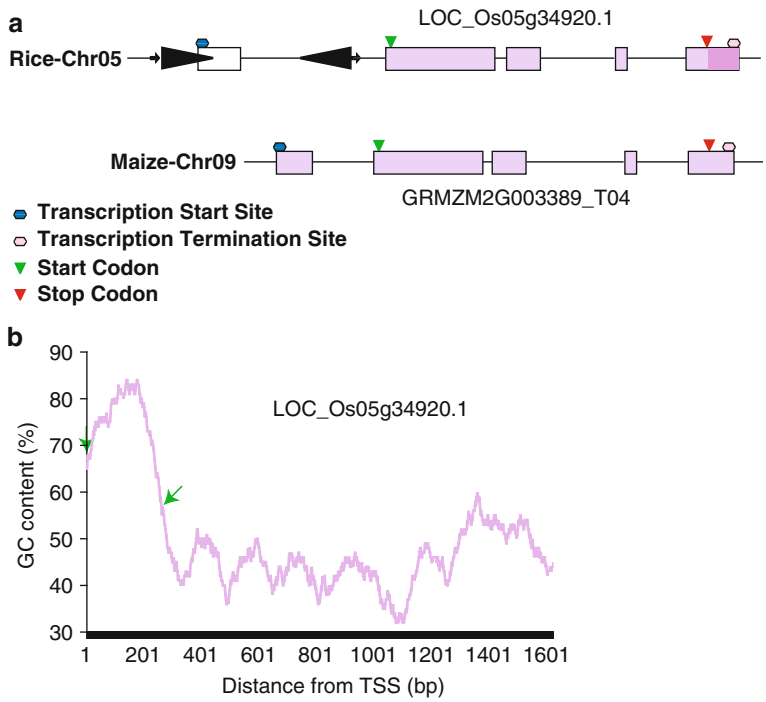

Fig. 5.3 Pack-MULEs involved in modification of adjacent genes. Pack-MULE TIRs are shown as *black arrowheads*, and *black horizontal arrows* indicate TSDs. Exons are depicted as *colored boxes*; other sequences are shown as *horizontal lines*. Gene annotations are based on MSU Rice Annotation Release 7.0 (http://rice.plantbiology.msu.edu/) and maize sequencing project (4a.53) (http://www.maizesequence.org/, B73 RefGen_v1). (**a**) A rice gene containing a novel 5′ UTR region derived from a Pack-MULE compared with its putative orthologs in maize. The rice gene structure is supported by a full-length cDNA. (**b**) The GC content, as the function of position from TSS (transcription start site), in the Pack-MULE-related transcript in A. The *green arrows* delimit regions contributed by the Pack-MULE

retrotransposon, unlike a class II element that can excise and transpose in another location, is to mutate and accumulate deletions, but it will remain at the same location until its complete elimination. This has been exploited to develop molecular markers which are powerful to trace the recent evolutionary history of a species or a population and was first proposed by the group of Okada, who used SINEs insertion to elucidate the phylogenetic origin of whales [86]. One of the specificity of the LTR retrotransposons in comparison with other class I elements, such as LINEs or SINEs, is that the sequence divergence between two LTRs can be translated into an insertion date, because, at the time of insertion, the LTRs of a single element are identical in sequence [87]. While common insertions between two phylogenetic groups provide a very strong information regarding common ancestry (like in the case of LINEs and SINEs), insertion dates provide additional information regarding the timing of TE-related genomic changes, thus providing some hint on the dynamics of phylogenetic differentiation. In rice, LTR retrotransposons were used to unravel the origin of Asian cultivated rice, i.e., the two distinct domestication events that gave rise to the two subspecies *indica* and *japonica* [26] and the close phylogenetic relationships between the cultivated species *O. sativa* and *O. glaberrima* [88, 89]. With the advent of the NGS, one could expect that the rice community will have a free access to the genome sequence of many accessions and can extend the scope of genomic paleontology surveys to many other varietal groups of the cultivated species or other species in the genus.

8 Horizontal Transfer of TEs in *Oryza*

TEs are often described as endogenous mobile DNA sequences. In this regard, LTR retrotransposons are often compared to retroviruses, with which they share several structural and functional features, such as long terminal repeats, polyprotein genes domain, primer binding site (PBS), and poly-purine tract (PPT). However, LTR retrotransposons generally lack one essential structural component that allows retroviruses to complete their life cycle, i.e., an envelope gene (*ENV*). First thought to derive from retroviruses by a loss of the *ENV* gene, LTR retrotransposons have been shown to actually predate retroviruses, the latter having acquired the *ENV* gene [90]. Over the past few years, many reports have demonstrated the efficiency of the epigenetic pathways that control transposition at large in eukaryotic genomes. In fact, as mentioned earlier in this chapter, most TEs families found in the rice genome appear to be transcriptionally and (therefore) transpositionally inactive. This, combined with the very fast elimination force that most TEs undergo posterior to their insertion in the genome, should lead to their extinction from most genomes, which is exactly the opposite of what is observed: from all the eukaryotic genomes fully sequenced so far, only two were found devoid of TEs, i.e., that of the *Plasmodium falciparum* [91] and that of the microalgae *Micromonas pusilla* [92]. All the others have a varying TE content, and TEs are often the major components of the genomes of both plants and animals. Far from being in extinction phase, TEs can be considered as one of the best success story in evolution. How TEs evade silencing is probably the next central question in genomics. One hypothesis has recently emerged for a possible explanation of TE survival and success in eukaryotes: horizontal transfers may allow TEs to move from one genome where they are targeted by silencing pathways, to another, devoid of this particular TE and where, therefore, it could propagate, until it is sensed and thus in turn targeted by the pathways in this new host genome. This working hypothesis is not easy to test in vivo, but in silico analyses can provide quantities of indirect evidences for such transposable elements-horizontal transfers (TE-HT). The first evidence of a TE-HT was provided by Diao et al. who showed that a MULE was transferred between *Setaria* and rice [93]. In addition, some recent results of Roulin et al., in two successive studies, indeed provide such evidence for the occurrence of TE-HT within the genus *Oryza* [94] and within Poaceae [95] (Fig. 5.2).

9 Concluding Remarks

The success of TE study in rice can be attributed to a variety of factors. First, rice is the most important crop plant worldwide and is associated with a large amount of genetic and genomic resources. Rice is the first crop plant whose genome was completely sequenced, and the high quality of genomic sequence has not been reproduced in any other crops. The availability of genomic sequence conferred unique advantage to the TE study in rice and enabled researches that are not possible for other plants. Second, about 45 % of the rice genome are composed of TEs, which is much more abundant than that in Arabidopsis, the most widely used model plant the genome of which was first sequenced in the plant kingdom. As a consequence, rice is a much better model organism for studying TEs than Arabidopsis. Third, rice has more than 20 wild relatives and their genomes are completely or partially available. Such resources provides basis for studying the evolution dynamics of TEs in rice. Finally, relatively easy transformation and regeneration of rice make it possible to conceive experiments that are impossible to conduct in plants such as maize. With such unique advantages, rice is anticipated to be the continuing model organism for plant TE studies.

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Molecular Cytogenetics of Rice and Its Wild Relatives

6

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1 Introduction

The genus *Oryza* is divided into 24 species based on morphological, cytological, biochemical, and molecular evidence including two cultivated species, *O. sativa*, Asian cultivated rice, and *O. glaberrima*, African cultivated rice, and 22 wild species represented by 10 genome types [1] (Table 6.1). Rice (*O. sativa*) is one of the most important staple crops in the world, and thus a number of cytogenetic studies have been done in rice. In recent decades, molecular cytogenetics, which combines molecular techniques and cytogenetics, has contributed to our understanding of chromosome and genome structure, phylogeny, and genome evolution in the *Oryza* species. Fluorescence in situ hybridization (FISH) has been an essential tool in molecular cytogenetics to visualize unique and repetitive DNA sequences on chromosomes using epifluorescence microscopy. FISH uses labeled nucleotides incorporated into DNA sequences, called a probe, to hybridize to complementary chromosomal DNA sequences. The probe is detected as a signal by a fluorescent microscope. FISH targets include interphase nuclei, somatic chromosomes, meiotic chromosomes, and extended DNA fibers. Recent advances in FISH allow one to distinguish and identify chromosomes, integrate genetic maps with specific chromosomes, compare physical and genetic distances, analyze distribution of repetitive DNA throughout a genome, associate genes or specific DNA sequences to chromosomal positions on chromosomes (Fig. 6.1), and determine genome origins in hybrids.

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2 Identification and Cytological Analysis of Individual Chromosomes of Rice

Distinguishing individual chromosomes of rice is very difficult due to their small size, similar morphology, and the lack of reliable banding patterns [2]. However, identification of rice chromosomes was achieved by quantitative analysis of uneven condensation patterns (CP) that appear on mitotic prometaphase chromosomes. Based on CPs and centromeric positions, 332 (92.2 %) out of 360 chromosomes were automatically identified, indicating that CP is a reliable and useful approach for chromosome identification [3]. A quantitative chromosome map of 12 rice chromosomes was developed using a chromosome image analyzing system (CHIAS), showing condensation patterns, total chromosomal length, and arm ratio [4, 5].

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Table 6.1 Summary of 24 species of the genus *Oryza*

Species	Chromosome number	Genome type	Genome size (Mb)	Reference
O. sativa	24	AA	389	[50]
O. nivara	24	AA	448	[51]
O. rufipogon	24	AA	439	
O. glaberrima	24	AA	357	[51]
O. longistaminata	24	AA	734	[52]
O. barthii	24	AA	611	[52]
O. meridionalis	24	AA	489	[53]
O. glumaepatula	24	AA	489	[53]
O. punctata	24	BB	425	[51]
O. officinalis	24	CC	651	[51]
O. rhizomatis	24	CC		
O. eichingeri	24	CC	562	[54]
O. minuta	48	BBCC	1,124	[51]
O. malapuzhaensis	48	BBCC		
O. latifolia	48	CCDD	1,125	[54]
O. alta	48	CCDD	1,008	[51]
O. grandiglumis	48	CCDD		
O. australiensis	24	EE	965	[51]
O. ridleyi	48	HHJJ	1,283	[51]
O. longiglumis	48	HHJJ		
O. granulata	24	GG	882	[51]
O. meyeriana	24	GG		
O. brachyantha	24	FF	362	[51]
O. coarctata	48	HHKK		

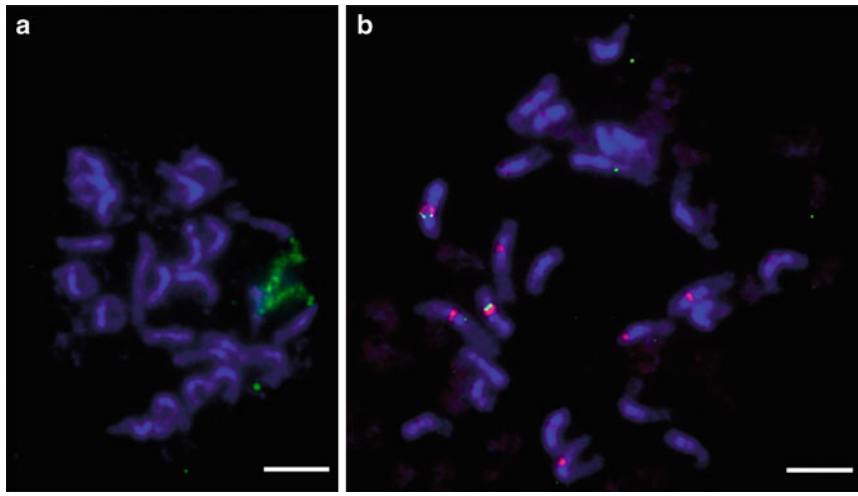

Fig. 6.1 Use of FISH to detect ribosomal DNA on mitotic prometaphase chromosomes of *O. sativa* L. ssp. *japonica* cv. Nipponbare. Chromosomes were counterstained with DAPI (*blue*). (**a**) 45S rDNA is detected on chromosome 9 as *green* signals. (**b**) 5S rDNA and BAC B1109A06 containing CentO repeat are detected on chromosome 11 as *green* and *red* signals, respectively. Bars represent 5 μm

FISH using chromosome-specific bacterial artificial chromosome (BAC) clones is also a powerful tool to identify chromosomes. Cheng et al. [6] used 24 chromosomal arm-specific BAC clones as probes for FISH to identify chromosomes at the meiotic pachytene stage. Pachytene chromosomes have higher resolution and more distinct cytological characteristics such as heterochromatin, euchromatin, and centromeres. The heterochromatin distribution pattern is highly conserved between *japonica* and *indica* subspecies, and an ideogram of pachytene chromosomes with the distribution of heterochromatin was developed based on the staining pattern of 4′,6-diamidino-2-phenylindole (DAPI) in *japonica* subspecies [6].

3 Physical Mapping of Low- or Single-Copy Sequences on Rice Chromosomes

Physical mapping of low- or single-copy sequences using FISH is an effective way to know the precise location of the sequences on chromosomes and their relation to chromosomal cytological features. In plants, the minimum length of detectable probes is around 1–3 kb [7–9]. However, the use of small probes has issues with low frequency of detection (e.g., distinguishing hybridization signals from background fluorescence noise) as well as inconsistency of the results [10]. To overcome this obstacle, large-insert genomic clones containing small target DNA sequences have been used successfully to map low- or single-copy DNA sequences in many plant genomes. For example, in rice, several BAC clones closely linked to a specific gene such as *Pi-b*, *Xa-21*, and *Pi-ta* [2] were successfully mapped onto mitotic chromosomes using FISH [7, 11, 12].

The integration of genetic and cytological maps is essential to understand chromosome structure and recombination frequency along chromosomes and to determine the precise location of target DNA sequences. Genetic maps do not reflect the actual physical distance across chromosomes because recombination is a nonrandom process across each chromosome. FISH mapping using large DNA clones containing genetic markers is useful to integrate cytogenetic and genetic maps. The rice genetic and cytogenetic maps have been integrated via 24 chromosomal arm-specific BAC clones containing RFLP markers [6]. Eighteen BAC/PAC clones containing RFLP markers were physically mapped onto rice pachytene chromosomes 10 and 5, respectively, to show the precise localization of each BAC clone on pachytene chromosomes. The comparison between genetic and FISH-based cytological maps illustrated the uneven distribution of genetic recombination along entire chromosomes [13, 14].

4 Repetitive Sequences Maintaining Chromosomal Structure and Function in the Genus *Oryza*

Plant genomes are composed of a significant fraction of repetitive sequences including tandem and dispersed repeats. Some of these repeats are responsible for maintaining chromosomal structure and function such as centromeres, telomeres, and other heterochromatic regions. Transposable elements—DNA transposons and RNA transposons (i.e., retrotransposons)—exist in all species of the genus *Oryza*. Among these elements, long terminal repeat (LTR) retrotransposons are the most abundant. LTR retrotransposons can increase the genome size of an organism in a relatively short time period because of their replicative mode of transposition and large element size. It is known that recent amplifications of LTR retrotransposons have contributed to the genome expansion of both *O. australiensis* (EE) and *O. granulata* (GG) resulting in genome sizes more than twice the size as all the AA genome species, including *O. sativa* [15]. The abundance and distribution patterns of LTR retrotransposons differ among families; some retrotransposons are dispersed genome wide, whereas others are concentrated in heterochromatic regions, such as centromeres, pericentromeres, or telomeres [16].

Centromeres are critical sites for sister chromatid cohesion and kinetochore assembly. Centromeres become visible as primary constrictions on mitotic and meiotic chromosomes.

Centromeres in plant genomes are often organized into megabase (Mb)-sized blocks, consisting mostly of repetitive sequences, such as satellite tandem repeats and retrotransposons. Because of the density of repetitive sequences, centromere organization is still poorly understood even in sequenced higher organisms. Rice is one of the few species where centromere organization is understood due to the use of cytogenetics to assist in the sequencing of several rice centromeres.

Rice centromeres primarily consist of two types of repetitive sequences, centromeric retrotransposons in rice (CRR), and centromere-specific tandem repeats (CentO [17–19]). CRR elements belong to the Ty3-gypsy superfamily, and FISH confirmed that they are enriched in centromeric regions. Unlike most plant LTR retrotransposons, CRRs are highly conserved in the genus *Oryza* and across the *Poaceae* family [17]. Southern blot results indicated that CRRs were present in almost all species of the genus *Oryza*, including the most distant relative of rice, *O. granulata* [20]. One exception is *O. brachyantha* (FF) where CRR elements were absent and their role has likely been replaced by another LTR retrotransposon named FRetro3 [20].

In contrast to centromeric retrotransposons, centromere-specific tandem repeats are variable within and between species of the genus *Oryza*. CentO is present in the AA, BB, CC, BBCC, CCDD, and EE genomes but not in the FF genome [19, 21, 22]. In *O. officinalis* (CC), the amount of CentO is quite low and another satellite repeat, CentO-C, is the dominant tandem repeat. FISH results showed that CentO exists at centromeres only in one pair of chromosomes in *O. officinalis* (CC), and the other ten centromeres contain CentO-C [23]. In *O. brachyantha* (FF), CentO has been completely replaced by CentO-F at all 12 centromeres and has no sequence similarity with CentO [21].

The size and intensity of FISH signals of repetitive sequences are correlated with the relative abundance of the repetitive sequences at their positions. Based on FISH signals of CentO in rice pachytene chromosomes and fiber-FISH results, the amount of CentO in the 12 chromosomes was estimated to range from 60 kb to 2 Mb [19] in size. Centromere 8 is the smallest and was the first completely sequenced centromere from any multicellular eukaryote [24, 25]. The amount of CentO dramatically changes from species to species, even in orthologous chromosomes (Fig. 6.2).

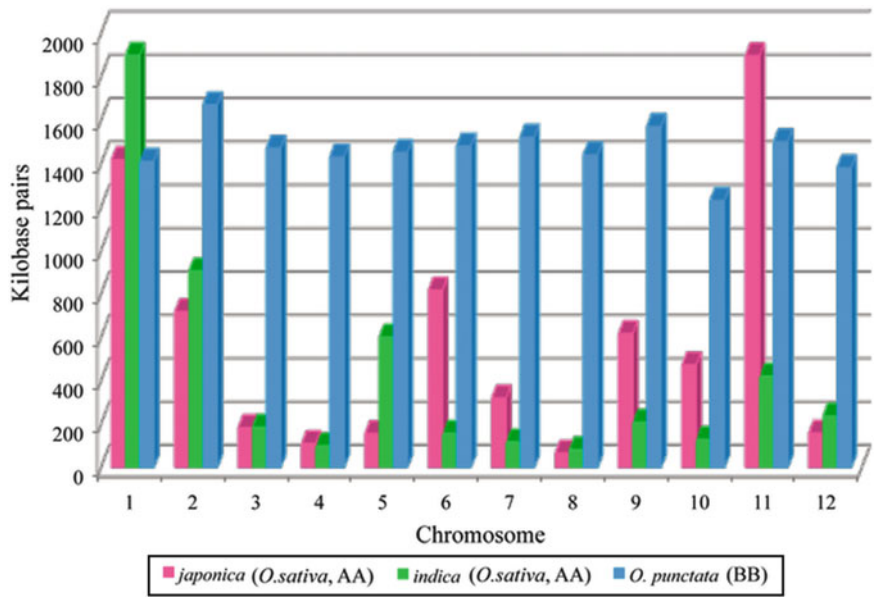

Fig. 6.2 The amount of CentO repeats in three genomes from two subtypes of *O. sativa* (AA) and *O. punctata* (BB) [19, 22]

A question arises as to why the amount of CentO is so different between orthologous *Oryza* chromosomes. Recently, 87 kb of CentO sequence in centromere 8 from the Kasalath variety (*indica* subspecies) was fully sequenced, and the result suggested that CentO was likely amplified in Kasalath after the divergence of subspecies *japonica* and *indica* [26]. However, the difference in size of CentO repeats can be more than fivefold between orthologous centromeres of *japonica* and *indica*. The simple sequence amplification model may not fully explain such a large difference after the divergence of these two subspecies from their common ancestor. Deletions of the CentO sequence in one species may have accompanied expansion in the other.

Telomeres serve as physical caps to protect the ends of chromosomes from degradation and fusion with each other. So far, telomere sequences from more than 120 eukaryotes have been identified and are included in the Telomere Database [27]. The telomeric repeat, TTAGGG, is highly conserved in vertebrates, invertebrates, plants, and some fungi. The TTTAGGG motif is abundant in telomeric regions of rice and other species of the genus *Oryza* [28]. The amount of telomeric repeats is variable among different chromosomes. Mizuno et al. [28] estimated that rice telomeres ranged in size from 5.1 kb in chromosome 7L to 10.8 kb in chromosome 6L by using a combination of the terminal restriction fragment (TRF) southern blot and fiber-FISH methods. In addition to telomeric repeats, 355-bp TrsA tandem repeats were identified in subtelomeric regions of rice and other *Oryza* species. Not all subtelomeric regions contain TrsA sequence and the TrsA-block sizes vary among rice varieties [29]. The function and origin of subtelomeric repeats are not clear, but it was proposed that they may act as buffer sequence to block the spread of gene silencing at telomeric positions [30]. Recent FISH mapping study of subtelomeric repeats in *Oryza* species provided interesting insight into the origins of centromeric and subtelomeric repeats. TrsC repeats, which are exclusive to the CC genome, were detected at multiple subtelomeric regions of *O. officinalis* (CC); however, the same repeats had multiple subtelomeric and centromeric loci in *O. rhizomatis* [23] (CC).

5 Applications of Extended DNA Fiber FISH

Extended DNA fiber FISH (fiber or EDF-FISH) is an improvement of FISH mapping in terms of spatial resolution down to a few kilobases and detection sensitivity up to 700 bp [31–33]. EDF-FISH has multiple applications including estimation of physical length and copy numbers of repetitive sequence and physical gaps between adjacent sequences (e.g., Fig. 6.3). EDF-FISH using telomere sequences and TrsA revealed the structure of the ends of chromosomes 6 and 12 of rice including the distance between telomere sequence and TrsA, their total lengths, and copy numbers [28, 34, 35]. EDF-FISH also revealed the intermingled structure between CentO and CRR [17, 19]. In the rice sequencing project,

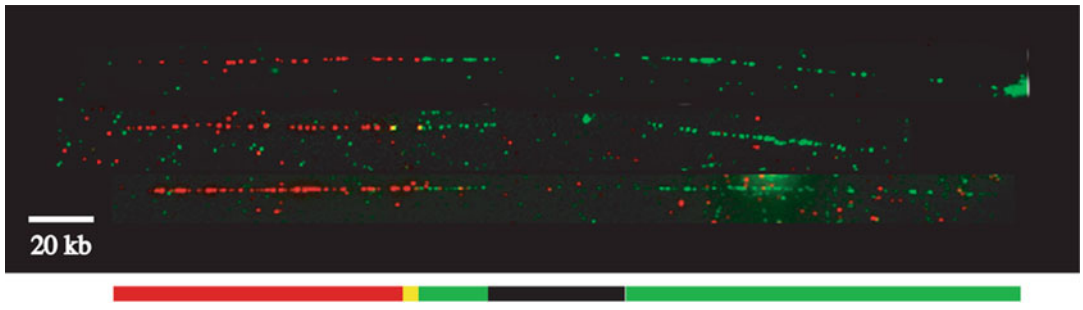

Fig. 6.3 Fiber-FISH of BAC clones 23I19 (*green*) and 92N12 (*red*) in *O. sativa* L. ssp. *japonica* cv. Nipponbare. *Yellow* fluorescence results from overlapping signal. A gap approximately 47.2 ± 14.9 kb in size consistently appears in the hybridization pattern of 23I19

EDF-FISH was successfully used to determine the size of unsequenced gaps for chromosomes 1, 4, and 10 [36–38].

6 GISH in *Oryza* Species

Genomic in situ hybridization (GISH) uses a total genomic DNA from one species as a probe to hybridize with target chromosomal DNA. The extent of hybridization depends on sequence identity or divergence between probe DNA and target chromosomal DNA. GISH allows one to identify the genomic contribution in interspecific hybrids and polyploids, alien chromosomes, introgressed chromosomes and chromosomal regions, and the cytogenetic relationship among related genomes. Table 6.2 summarizes the reports of GISH experiments in a variety of *Oryza* species. These studies show successful GISH experiments in polyploid *Oryza* species, indicating that their genomes are sufficiently diverged to be distinguished using GISH. The extent of GISH hybridization on *O. latifolia* (CCDD) and *O. minuta* (BBCC), using *O. officinalis* (CC) as a probe, indicates that the C and D genomes are more closely related to each other than the B and C genomes [39].

GISH can also be useful for breeding. GISH has been used to reveal the genomic constitution, including introgressed or translocated regions of chromosomes of interspecific hybrids and derived lines [40–44].

Table 6.2 GISH studies in the *Oryza* species

Target chromosomal DNA	Probe DNA	Reference
O. latifolia (CCDD)	*O. officinalis* (CC)	[39]
O. minuta (BBCC)	*O. officinalis* (CC)	[39]
Somatic hybrid of *O. sativa* (AA) × *O. punctata* (BBCC)	*O. sativa* (AA), *O. officinalis*(CC)	[39]
F1 hybrid of *O. sativa* (AA) × *O. eichingeri* (CC)	*O. eichingeri* (CC)	[41]
F1 hybrid of *O. sativa* (AA) × *O. meyeriana* (GG)	*O. sativa* (AA), *O. meyeriana* (GG)	[44]
Monosomic alien addition line (MAAL) of *O. officinalis* to *O. sativa* and its backcross progenies	*O. officinalis* (CC)	[42, 43]

7 Detection of Epigenetic Modification on Chromosomes

Heterochromatin plays a significant role in the suppression of genes and transposable elements as well as the maintenance of chromosome structure. The epigenetic networks of molecular interactions, including DNA methylation, histone modification, and recruitment of protein complexes, are associated with heterochromatin formation and maintenance. Several studies have shown the association of particular epigenetic modifications with gene silencing and heterochromatin. For example, in *Arabidopsis*, dimethylated histone H3K9 has been shown to be a critical mark for DNA methylation and gene silencing [45]. Both methylated DNA and dimethylated histone H3K9 are also prominent epigenetic marks for heterochromatic regions, indicating their potential roles in heterochromatin formation and maintenance [46]. Rice has the patterns of epigenetic modifications consistent with *Arabidopsis*. Methylcytosine immunoprecipitation (mCIP) and immunostaining using an anti-5-methylcytosine antibody revealed that the chromosomal distribution of DNA methylation is coincident with heterochromatin on rice meiotic chromosomes [47]. Immunostaining using anti-dimethylated H3K9 showed that dimethylated H3K9 signals were detected along rice pachytene chromosomes, especially at pericentromeric regions, consistent with the DNA methylation pattern on rice pachytene chromosomes (Fig. 6.4) [48]. Figure 6.4d shows a pachytene chromosome where the signal of dimethylated H3K9 is enriched at condensed regions within each chromomere.

8 Conclusion and Prospectives

Rice has been and continues to be a model system for genetic, genomic, and cytogenetic studies. With the availability of genetic resources

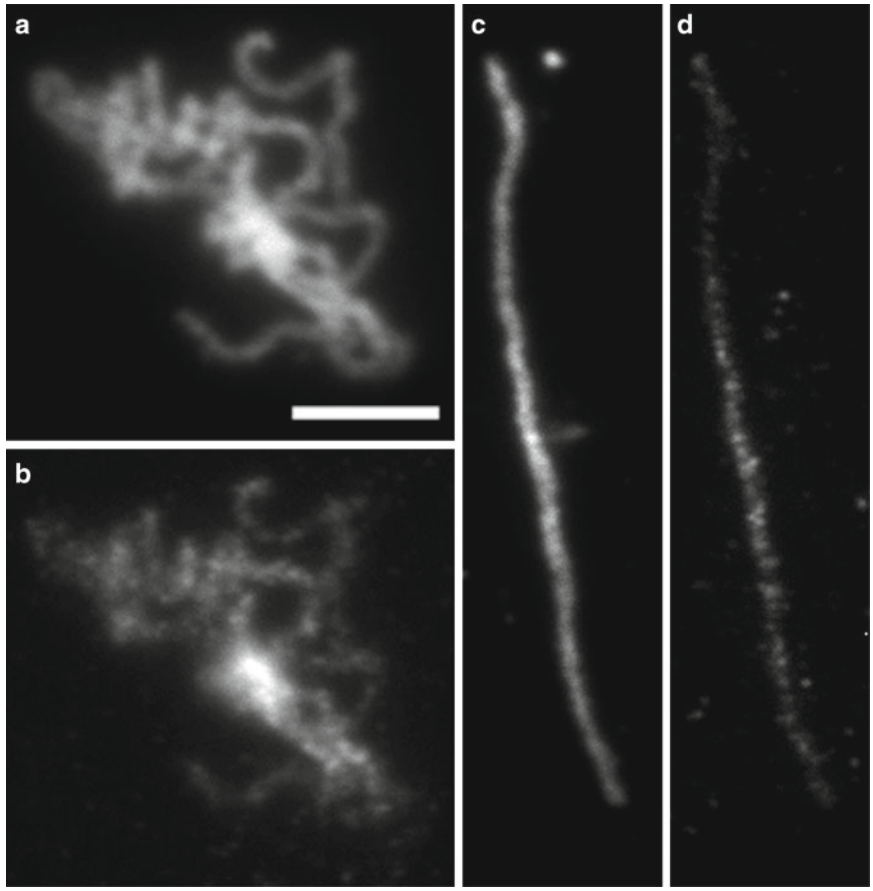

Fig. 6.4 Immunostaining results of histone dimethylated H3K9 [48]. This image is modified from Iwata et al. (**a**) Meiotic chromosomes at early pachytene stage counterstained with DAPI. (**b**, **d**) Signals of dimethylated H3K9. (**c**) A pachytene chromosome counterstained with DAPI. Bar represents 5 μm

spanning the entire genus [49], studies have led to greater knowledge of chromosomal structure and behavior, positions of interesting genes, centromere structure and function, epigenetic modifications, and chromosomal evolution in the genus *Oryza*. The development of FISH techniques and their applications have clearly broadened our fundamental knowledge of chromosomal biology. The combination of molecular cytogenetics and high-throughput sequencing techniques allows one to explore new facets of chromosome behavior and the effect of chromosome structure and positioning on gene function and patterns of epigenetic modifications, associated with chromosomal structures. Molecular cytogenetics research in the wild relatives of rice, spanning 15–20 MY of evolution, will illuminate the effects of evolutionary pressures on chromosome structure and behavior. We expect that the primary component of plant genomes, retrotransposons, will be a major modeling component of chromosomes, but it remains to be seen either at a chromosomal level or even at a genome level how these genomic "parasites" affect chromosomal and genetic adaptation and evolution.

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Mutant Resources for Functional Analysis of the Rice Genome

7

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1 Importance of Mutant Resources for Functional Analysis of the Rice Genome

The first high-quality sequence of a crop genome ever established was for rice [97], making rice, the first cereal of human consumption, the pivotal species for comparative genomics in monocotyledons [6, 52, 57]. Because of the importance of rice in human nutrition, the next challenge is to unravel the function of most genes underlying traits of agronomic importance by 2020 to assist in breeding cultivars with improved performance [159]. The most straightforward way to gain access to the function of a gene is to inactivate its expression and observe the alteration in phenotype. In rice, inactivation of gene expression has been achieved in a sequence-specific manner through posttranscriptional [145, 146] or transcriptional [85, 86, 88] gene silencing and gene targeting [45]. Mutant populations with random inactivation have been generated through physical [9, 150], chemical [140], or insertional mutagenesis (for review see [69]).

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The generation of insertion mutant collections was initiated in the late 1990s at the National Institute of Agrobiological Sciences (NIAS, Japan) and at the Pohang University of Technology (POSTECH, Korea) and involved the endogenous *Tos17* retro-element and T-DNA, respectively. These two laboratories have generated large collections of insertion lines (50,000 and 105,000 lines, respectively) that have been extensively shared among international research groups to allow for deciphering the function of many genes through forward and reverse genetics strategies (see Sects. 4 and 5). Soon after, initiatives of T-DNA insertion mutagenesis were launched in France (Génoplante consortium of public institutions CIRAD-INRA-CNRS-IRD and private companies Biogemma and Bayer Crop Science), China (a consortium of Huazhong Agricultural University, Zhejiang University, the Biotechnology Research Institute and the Shanghai Institute of Plant Physiology and Ecology) and Taiwan (Institute of Plant and Microbial Biology and Institute of Molecular Biology, Academia Sinica; and the Taiwan Agricultural Research Institute), which have generated 370,000 additional lines. In parallel, *Ds* and *Dspm* maize transposable elements have been introduced in rice on a large scale to create lines carrying transposon launching pads in Korea (PMBBRC), Australia (CSIRO), Europe (EU OSTID consortium), Singapore (Temasek), and the United States (UC Davis), for 150,000 lines (Table 7.1).

An important seed increase from the generated lines under field conditions has been achieved, which also provide information on the range of phenotypic variation. Specific screens for alterations in morpho-physiological traits have been carried out. As to the reverse genetic approach, a line harboring an insert disrupting a gene of interest (GOI) in the collections was first identified in mutant DNA pools by PCR with GOI- and insert-specific primers. However, the systematic sequencing of genomic regions adjacent to the mutagen insertion sites (flanking sequence tags: FSTs) now allows a search based on the resulting sequence information released in GenBank and made accessible in dedicated genome navigators, such as RICE-GE http://signal.salk.edu/cgi-bin/RiceGE or OrygenesDB http://orygenesdb.cirad.fr/. A significant portion of the 675,000 insertion lines has been used to generate 240,000 FSTs (Table 7.2). Still paralleling the insertion mutagenesis effort, conventional mutant populations have been generated by treating seeds with chemical or physical mutagenic agents, first at UC Davis and IRRI, in japonica and indica rice, respectively. The possibility to use reverse genetics routinely with these populations, through DNA arrays, TILLING on gel or by sequencing, has intensified the effort to generate mutant collections in many countries.

The steep increase in number of publications describing the use of rice insertion mutants for unraveling gene function in the last 5 years clearly indicates the power of these collections. Here, we review the current status of mutant collection and characterization, the actual genome coverage by inserts and lesions, the advantages and disadvantages of each mutagen learned from experience and the range of traits that have been functionally analyzed by using these resources. Finally, we will describe current trends in mutant analysis for unraveling the function of all rice genes underlying traits of agronomic importance by the end of this decade.

2 Available Mutant Resources and Related Sequence and Phenotype Information

2.1 Physical Deletion Mutants (γ-Rays, Neutron) and Chemical Point Mutants (EMS, SA, MNU)

Genomic deletions induced by irradiation and chemical mutagens provide a rapid way to obtain large mutant populations. Use of gamma rays and fast neutrons to produce deletions of variable

Table 7.1 Current status of rice mutant collections and associated resource databases

Library	Institution(s)	Genetic background	Mutagen(s)	Number of lines produced	Number of lines seed increased for distribution	Number of lines with phenotype information	Reference publications related to the resource	Order website (contact person)
POSTECH Rice Insertion Database (RISD)	Pohang Univ. of Technology and Kyung Hee Univ., Korea	Dong Jin Hwayoung Kitaake	T-DNA GT, AT	105,000	75,000	nd	[4, 48, 49, 50, 79, 126]	http://signal.salk.edu/cgi-bin/RiceGE genean@khu.ac.kr
Rice Mutant Database (RMD)	Huazhong Agricultural Univ., Zhejiang Univ.,	Zhonghua 11 Zhonghua 15 Nipponbare	T-DNA ET *Tos17*	134,346 100,000	12,000	31,755	[12, 91, 148, 157, 158]	http://rmd.ncpgr.cn/ cywu@mail.hzau.edu.cn
Taiwan Rice Insertion Mutant (TRIM)	Academia Sinica, Taiwan	Tainung 67	T-DNA AT	90,000	75,000	75,000	[14, 44]	http://trim.sinica.edu.tw bohsing@gate.sinica.ed.tw
Oryza Tag Line (OTL) Génoplante	CIRAD-INRA-IRD-CNRS, France	Nipponbare	T-DNA ET (+*Ds*) *Tos17*	31,000	25,000	25,000	[58, 73, 93, 123, 128, 129]	http://oryzatagline.cirad.fr/ emmanuel.guiderdoni@cirad.fr
Shanghai Insertion Population (ShIP)	SIPPE, China	Zhong hua 11	T-DNA ET	10,000	nd	nd	[26]	http://ship.plantsignal.cn/home.do ship@sibs.ac.cn
Tos17 insertion database	Nat. Inst. of Agrobiological Sciences (NIAS), Japan	Nipponbare	*Tos17*	50,000	50,000	50,000	[98, 99, 100, 101, 153]	http://tos.nias.affrc.go.jp hirohiko@nias.affrc.go.jp
CSIRO	CSIRO Plant Industry (Australia)	Nipponbare	Ac/*Ds* GT/ET	5,000	nd	nd	[23, 143]	http://www.pi.csiro.au/fgrttpub narayana.upadhyaya@csiro.au
EU OSTID	European Consortium	Nipponbare	Ac/Ds ET	5,000	5,000	nd	[20, 33, 144]	http://orygenesdb.cirad.fr/ emmanuel.guiderdoni@cirad.fr
Temasek Ds	Temasek Life Sciences, Singapore	Nipponbare	Ac/Ds GT	20,000	nd	nd	[54, 68]	sri@tll.org.sg
UC Davis Ac/Ds and En/Spm populations	UC Davis, USA	Nipponbare	Ac/Ds GT En/Spm Ac/Ds AT	20,000	nd	nd	[68, 71, 125]	http://www-plb.ucdavis.edu/Labs/sundar/ sundar@ucdavis.edu

(contiuned)

Table 7.1 (continued)

Library	Institution(s)	Genetic background	Mutagen(s)	Number of lines produced	Number of lines seed increased for distribution	Number of lines with phenotype information	Reference publications related to the resource	Order website (contact person)
NAAS Rice Insertion Mutant Database (NRIMD)	Nat. Academy of Agricultural Science, RDA, Korea	DongJin	Ac/Ds GT	100,000	50,000	5,000	[15, 74, 76, 80, 83, 113, 117, 118]	
IR64 deletion mutant population	Internat. Rice Research Institute, The Philippines	IR64	Fast neutron γ-ray DEB, EMS	40,000 M2	nd	nd	[9, 19, 150]	
UC Davis TILLING population	UC Davis, USA	Nipponbare	SA, MNU	6,000 M2	6,000	nd	[140, 141]	http://tilling.ucdavis.edu/ lcomai@ucdavis.edu
NIAS mutant population	Nat. Inst. for Agrobiological Sciences (NIAS), Japan	Nipponbare	γ-ray ion beam	15,000 M2 7,000 M2	15,000 M2 7,000 M2	nd		nisimura@affrc.go.jp
Zhejiang mutant population	Zhejiang Univ., China	Kasalath SSBM	γ-ray EMS	40,000 M2	nd	nd		http://www.genomics.zju.edu.cn clspwu@zju.edu.cn

Table 7.2 Current flanking sequence tag information released in public databases: 92.4 % are anchored on the Unified Rice Pseudomolecules 7.0 release between the MSU Rice Genome Annotation Project and the Rice Annotation Project Database (RAP-DB)/International Rice Genome Sequencing Project

Mutagen	Source	No. of sequences	No. mapped	% Mapped	References
Ds	CSIRO	611	572	93.6	[143]
Ds	OSTID	1,380	1,301	94.3	[144]
Ds	PMBBRC	1,072	1,034	96.5	[74, 76, 80, 83, 117, 118]
Ds	UCD	4,841	4,419	91.3	[68]
Dspm	UCD	12,889	11,657	90.4	[71]
T-DNA	OTL	26,788	24,256	90.5	[93, 123, 128]
T-DNA	Postech	107,171	100,447	93.7	[4, 49, 79]
T-DNA	RMD	23,175	21,239	91.6	[157]
T-DNA	ShIP	12,614	8,875	70.4	[26]
T-DNA	TRIM	11,799	11,340	96.1	[44]
Tos17	OTL	14,284	13,874	97.1	[93, 123, 128]
Tos17	RMD	9,707	8,913	91.8	Long et al. Unpubl.
Tos17	NIAS	18,024	17,830	98.9	[101]
Total		244,355	225,757	92.4	

sizes (70–500 kb) allows for simultaneously knocking out genes tandemly repeated as two or more copies, estimated to represent 22 % of rice genes that sometimes display redundant functions. In rice, a population of 40,000 M3/M4 mutant lines has been established in the lowland cultivar IR64 as a result of various chemical and irradiation mutant induction strategies [150]. The population has been used for detecting morphological and physiological changes and altered pathogen and drought responses. The population is amenable to reverse genetics with various techniques [95]. A first technique, Delete-a-Gene®, relies on the preferential amplification and subsequent enrichment of smaller fragments created by deletions within the PCR products amplified from pooled DNA samples of mutagenized populations [89]. A second method relies on the hybridization of labeled genomic DNA directly onto the Affymetrix Rice GeneChip® and rapid localization of deleted regions in mutants. Use of this chip hybridization allowed for predicting deletions ranging from 1 gene model to ~500 kb on all 12 rice chromosomes [9]. The utility of the technique was demonstrated in a lesion mimic phenotype by the use of allelic series of mutants to rapidly narrow down the genomic region and eventually identify a candidate gene responsible for the phenotype [9]. The positions of mutations in 14 mutants were aligned onto the rice pseudomolecules to allow for rapid identification of untagged mutations. Such populations are of particular importance for analysis of the *indica* genome because it is less accessible to insertion mutagenesis than the *japonica* genome, owing to the overall poor tissue-culture response of cultivars in this subspecies.

Rice seeds mutagenized by different chemical mutagenic treatments (EMS, SA, and MNU) were found to harbor a suitable high density of lesions (≥1/500 kb), and M2 or M3 DNA samples are used for reverse @ or ca. 1/500 kb genetics by TILLING [16]. TILLING is based on the detection of a single nucleotide mismatch specifically recognized by the endonuclease CEL1 in heteroduplexes. Heteroduplexes are formed following denaturation and annealing of PCR products amplified with target gene-specific primer pairs in pooled DNA samples containing mutant and wild-type alleles of a target gene. Resulting shorter strands are detected differentially on a sequencing gel after appropriate labeling. The

TILLING method has been used with the model rice cultivar Nipponbare at the USDA ARS laboratory and UC Davis [140]. The ARS laboratory now provides a high-throughput TILLING service for rice genes that is open to the community. In total, 5,120 individuals have been arrayed and are screened as units of 512 individuals × 4 or ×2. A pilot study identified 318 mutations in 21 test target genes by scoring 28,900–1,600-bp amplicons. Recently, next-generation sequencing coupled with multidimensional pooling has been used to identify rare alleles in this mutant population. The development of the method required discriminating sequencing errors from real changes, a task that was facilitated by sufficient sequencing coverage and suitable bioinformatics analysis [141]. The combination of pooling strategies, PCR amplification, Illumina GA platform sequencing, and a bioinformatic pipeline that assigns probabilities to each candidate resulted in efficient detection with a low false-positive rate. M3 seed are distributed by the Dale Bumpers Rice Center in Stuttgart, Arkansas. Another MNU-mutagenized population has been developed in Japan [133].

2.2 T-DNA

Rice remains among the cereal species the most amenable to transformation [37], and high-throughput transformation procedures for rice functional genomics were established in the late 1990s. Optimized rice transformation procedures have efficiencies 5–20 times higher than those previously reported for *japonica* rice, with 1–10 transgenic plants produced per cocultured callus depending on the cultivar. Large populations of T-DNA insertion lines have been generated in the cultivars Dong Jin, Hwayoung, Nipponbare, Zhonghua 11, Zhonghua 15, and Tainung 67. T-DNA insertions are chemically and physically stable over generations, may carry powerful gene detection and/or activation systems (Fig. 7.1 and Sects. 5 and 6) and/or a *Ds* element and are phenotypically tagged through the expression of selectable (and possibly reporter) gene(s). T-DNA is integrated in low-copy number (average of two copies at 1.4 locus per line), thereby facilitating further genetic and molecular analyses but also rendering necessary the generation of large libraries to ensure genome saturation. Some drawbacks of T-DNA insertion mutagenesis are that the mutations found in these lines are frequently untagged because of somaclonal variation and also to lesions due to abortive integrations of T-DNA. Because of the often complex organization of T-DNA inserts, which includes concatemerized and/or truncated copies and/or binary vector sequences, sequencing of up to 40 % of flanking regions is unproductive and may create artefacts in enhancer trapping-mediated reporter gene expression.

2.3 *Tos17*

Tos17 is a *Ty1-copia* retrotransposon of rice belonging to a 32-member family, of which 5 members have been found active (*Tos10*, *17*, *19*, *25*, and *27*) [39]. *Tos17* is the most active and exists in low-copy (1–11) number in rice cultivars [39, 120], and its transposition is repressed at the transcriptional level during normal culture conditions. New copies resulting from a copy-and-paste mechanism of transposition accumulate with cell culture duration. New *Tos17* copies generally insert at unlinked loci from the endogenous active copy(ies) throughout the genome because of retrotranscription of template mRNA in the cytoplasm and further importation of cDNA to the nucleus for integration in chromosomes. More than ten new inserts have been observed per regenerated line, thereby limiting library size, and are stably transmitted to the progeny [38].

Recovery of genomic regions flanking insertion points is easy with the mechanism of insertion. However, the presence of resident copies and multiple new copies may further complicate dissociation of insertions during sequencing of flanking regions and molecular and genetic analyses. The shared origin of cells giving rise to regenerated plants leads to a redundancy of some copies, and sequencing may be unproductive (e.g., only 16,784 [40 %] independent sequences have been obtained from 42,292 sequenced products) [101].

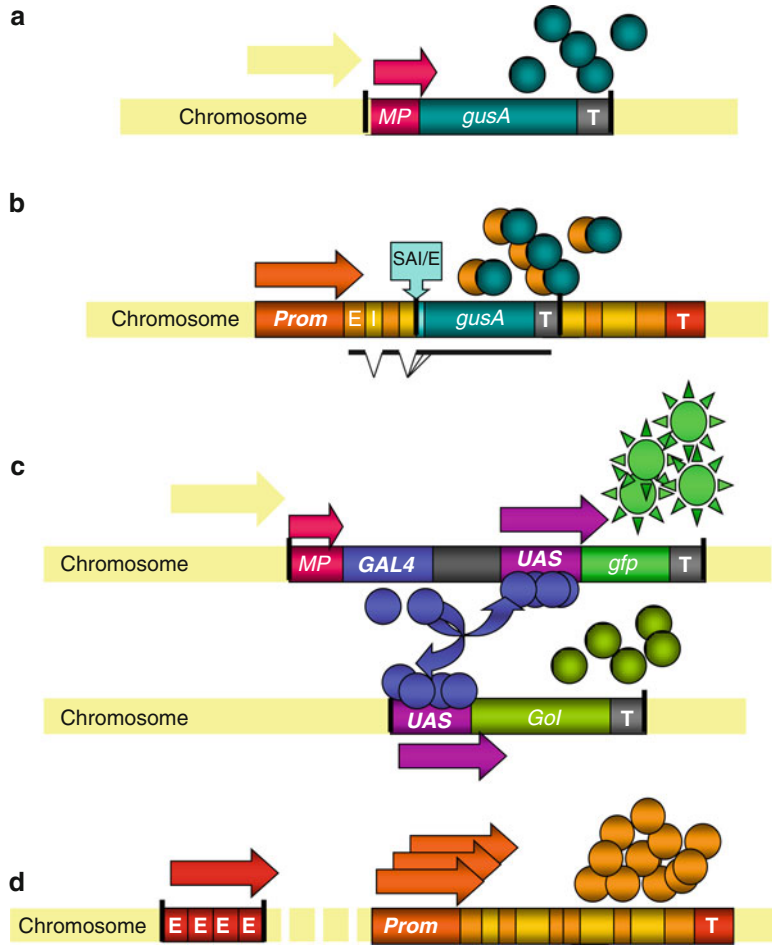

Fig. 7.1 Engineered insertional mutagens (T-DNA, *Ds*, and *dSpm*) may be equipped with powerful gene detectors and activators. (**a**) An enhancer trap (ET) comprises a reporter gene fused to a minimal promoter (MP) (e.g., –48 bp from the CaMV35S promoter, containing the TATA box and a transcriptional start site) that is not transcriptionally active but its transcription can be triggered by neighboring chromosomal enhancer elements. In rice, ET insertions tend to result in a high frequency of gene detection (20–30 %) but may not correspond to a disruption of the detected gene because ET can be influenced by enhancers residing in the insert or in genes positioned several kilobases away from the disrupted gene. For an illustration see [93]. (**b**) A gene trap contains a promoterless reporter gene expressed only when the insertion is within a transcriptional unit and in correct orientation. The presence of one or more splice acceptor sites aligned in all reading frames, preceding the reporter gene, allow expression if the insertion occurs in an intron. Reporter-gene expression patterns are generally observed at low frequency (5–10 %) but correspond to insertions within genes and more likely with knockouts. When insertion occurs in an intron or an exon, translational fusion can be generated between the reporter gene and upstream exons of the interrupted gene. This situation may create translational fusions, which provide information about protein localization. For illustrations see [15, 48]. (**c**) A modification of the classical enhancer trap includes a modified yeast transcriptional activator GAL4 gene (GAL4-VP16) fused to an MP and a gusA or a gfp gene fused to tandemly arrayed upstream activating sequences (UAS), which are recognized by GAL4 as binding sites. GUS activity or GFP detection reveals GAL4-VP16 expression because GAL4 controls the transcription of gusA or gfp through the UAS elements. After identification of a particular enhancer trap line with interesting gusA/gfp expression, a second construct can be introduced, with a GOI fused to UAS elements that will express only in tissues and cell types expressing gusA/gfp. For illustrations see [148, 58]. (**d**) An activation tag typically contains multimerized transcriptional enhancers (e.g., –343 to –90 fragment from the CaMV 35S promoter) conducting ectopic activation of expression of the neighboring gene as gain of function mutation. Transcription of genes near the AT insertion can be influenced up to 10 kb away. For illustrations see [44, 50, 125]

The utility of *Tos17* in reverse genetics has been exemplified in rice through the analysis of mutants altered in a large range of physiological and developmental processes (see Sects. 4 and 5). As in T-DNA insertion lines, among *Tos17* lines, a high frequency of untagged mutations (95 %) results from other sources of somaclonal variation during tissue culture. This limits the large-scale use of *Tos17* in forward genetics, although several genes have been discovered through mutant screens (see Table 7.6 and Sect. 5). The non-transgenic feature of *Tos17* lines is a clear advantage over T-DNA and *Ds* rice lines in terms of seed increase, evaluation under field conditions, and international exchanges.

Because the cultivars used for creating T-DNA insertion lines contain resident *Tos17* copies and the *Agrobacterium*-mediated transformation procedure includes tissue-culture steps, there is an advantage in characterizing new *Tos17* insertions in T-DNA plants. Nipponbare transformants contain an average of 3.5 new *Tos17* copies [123], whereas Tainung 67 transformants contain only 0.1 new copy per line [44]. T-DNA plants from Dong Jin and Zhonghua 11, which have been used for generating large collections of insertion lines, contain 4 and 1.4 new copies, on average, respectively. Large-scale sequencing of *Tos17* flanking sequences has been performed with the NIAS collection of regenerants and in OTL and RMD T-DNA lines.

2.4 *Ac*/*Ds* and *Spm*/*dSpm*

The pioneering work of Ko Shimamoto's group in the early 1990s demonstrated that the 4.565-kbp *Activator* (*Ac*) element originally isolated from maize was active in transformed rice protoplasts [46, 132]. Efforts were intensified in establishing gene machines with this system [24, 67]. A major problem with the autonomous *Ac* system is that transpositions of the element cannot be stabilized because of the continuous production of transposase (AcTpase), which catalyzes excision/reinsertion of the element. Moreover, multiple excision/reinsertion events across generations create excision footprint mutations that are no longer tagged by the element. Therefore, more sophisticated double-component systems with the transposase source dissociated from a nonautonomous *Ds* element that may include useful positive–negative selection markers are preferred. In these systems, the AcTpase source and immobilized *Ds* are introduced as linked on the same T-DNA [15, 24, 33, 68, 143] or separated on two T-DNAs and combined in the same line by crossing [68, 104]. In both cases, the *Ds* element and the AcTpase source can be segregated away from each other in subsequent generations.

The transposition behavior of *Ds* across generations of rice transformed by a single T-DNA/double-component approach has been detailed [33]. The frequency of transposition is usually high among primary regenerants (62 %), with early transpositions occurring during the transformation/regeneration process, which are generally incorporated in the germline, as well as late transpositions that can also be germinally inherited. Therefore, the advantage is to propagate callus tissue or ratoon primary transformants for generating more independent germinal insertions. The frequency of actively transposing families in the T1 generation is usually high (83 %), whereas retention of activity declines to 32 % in the T2 generation because of silencing of the *AcTpase* gene. However, the autonomous *Ac* element [67] or a *Ds* element mobilized in *trans* by an AcTpase source introduced by crossing [68] appear to be less prone to this silencing phenomenon. Early silencing of the *AcTpase* gene is favorable for stabilizing the inserts but not amplifying copies of the element, which may occur when the transposition takes place during replication, and would eventually allow for reduction in library size. Engineered *Ds* may be equipped with gene detection and/or activation systems (see Fig. 7.1 and Sect. 5).

Spm/*dSpm* is transcribed in rice. Although the first trial reported only somatic transpositions [33], with use of larger terminal inverted repeat (TIR) regions in the transformation construct, the system now works efficiently and produces germinally inherited insertions [71]. In the latter work, use of an elegant system led to the observation of high frequencies of unlinked germinal transposition of a nonautonomous, defective suppressor mutator (*dSpm*) for the first time in rice. The system is based

on a single T-DNA construct with Spm-transposase and a *dSpm* element combined with green fluorescent protein (GFP) and *Discosoma* sp. red fluorescence protein (*Ds*Red) fluorescent markers to select unlinked stable transpositions of *dSpm*.

An advantage of the transposon system over retrotransposons and T-DNA is that the element can be remobilized to generate a revertant phenotype, thereby avoiding the tedious complementation step. This system can be used to create new mutant alleles in a single gene or to locally saturate a target chromosomal region with insertions. However, non-transmitted somatic insertions and untagged mutations due to *Ds* excision footprints are important limitations of this system. Recovery of genomic regions flanking insertion sites is easy because of the clear cut-and-paste excision/reinsertion mechanism. Although variable, depending on the scheme used for stabilizing the insertions over generations, redundancy of sequenced flanking regions due to germinal sharing of inserts among sibling plants may also reduce the efficiency of large-scale FST generations [144]. Also, sequencing flanking regions from somatic insertions may lead to lack of transmission of the sequence-indexed insert in progeny seeds.

2.5 Other Insertion Mutagens

Two transposon systems have been characterized and suggested as alternative gene-tagging systems in rice: a 430-bp tourist-like MITE lacking the open reading frame (ORF), *mPing* (the first discovered DNA transposon active in rice), and another two-component transposon system of the hAT superfamily of transposons, *Dart*/*nDart*.

Miniature Ping (*mPing*) was identified in three independent studies through genome sequence analyses [53], analysis of mutability of the slender glume locus [106], and observation of its activation by anther culture [63]. The copy number of *mPing* in the *japonica* genome ranges between 60 and 80 from in silico and Southern analyses. This number is unusually low, with the copy number of other MITEs typically in the thousands. *mPing* appears to be a recent deletion derivative of a 5,353-bp element called *Ping*, consisting of an *mPing* sequence separated by two ORFs, the second most likely encoding a transposase. However, *Ping* appears not to be activated under the same conditions favoring *mPing* excision (anther culture and gamma ray irradiation), whereas another related element, called *Pong*, was indeed found to be active under conditions favoring *mPing* excision and would provide the transposase source in *trans* [53]. A reverse genetics search of DNA from 600 anther-derived calli for insertion in 20 sequences allowed for the identification of an insertion at the *waxy* locus [63], which suggests that the system is effective for gene tagging.

The 607-bp nonautonomous DNA-based active rice transposon (*nDart1*) element was identified as a causative element of a spontaneous mutable virescent allele, pyl-v, conferring pale-yellow leaves with dark green sectors in seedlings. This finding was related to the somatic excision of the nDart1 element from the OsClpP5 gene encoding the nuclear-coded chloroplast protease. The nDart has 19-bp TIRs and, when mobilized, generates an 8-bp target-site duplication (TSD) [28, 142]. The transposition of nDart1 can be induced by crossing with a line containing an autonomous element, aDart, and stabilized by segregating out of aDart by a mechanism similar to that of maize *Ac*/*Ds* [27]. The nDart/aDart system therefore appears to be the only endogenous rice DNA transposon system whose transposition activity can be controlled under natural growth conditions without any artificial treatments, including tissue culture, thereby avoiding somaclonal variation [108]. A survey of 19 temperate japonica, 30 tropical japonica, and 51 indica varieties showed that only 8 temperate japonica varieties harbored a single copy of an active aDart element, whereas no aDart activity could be detected in indica varieties examined [108].

3 Behavior of Rice Insertion Mutagens

High-throughput methods relying on inverse PCR, thermal asymmetric interlaced (TAIL) PCR, or adapter PCR have been used to amplify and

sequence 244,000 genomic regions flanking the insertion sites of T-DNA, *Tos17*, *Ds*, and *dSpm* mutagens in rice mutant populations (Table 7.2). Overall, nearly 226,000 sequence-indexed inserts have been positioned on the rice chromosome pseudomolecules (MSU v7.0 release). Several studies have detailed the insertion behavior of each of these mutagens over the rice chromosomes, and the outcome is summarized below.

3.1 T-DNA

Information on ~165,000 unique, insertion positions of T-DNA inserts is available for rice (Table 7.2). This sequence information was retrieved from T-DNA integration events that occurred in genomic regions not preventing the subsequent expression of the T-DNA-borne selectable marker gene, thereby introducing a possible bias over the actual behavior of the T-DNA. In other words, the requirement for expression of the selectable marker gene may introduce a bias against the recovery of T-DNA insertions in lowly transcribed, heterochromatic regions of the genome.

Consistent conclusions on the insertion behavior of the T-DNA were drawn from independent insertion mutagenesis programmes that made use of different genotypes, transformation, and FST production methods [34]. T-DNA integration density was generally higher in the largest chromosomes, which also exhibit the highest transcribed gene densities (Table 7.3). Interestingly, the correlation of gene density and insertion density among the 12 chromosomes is stronger for insertion of T-DNA ($r2=0.94$) than *Tos17* ($r2=0.42$). *dSpm* and *Ds* exhibit intermediate correlations (Table 7.3). T-DNA insertions are not evenly distributed on each chromosome: insertion frequencies are high at the distal, subtelomeric regions and low in regions close to the centromeres [4] (illustrated in Fig. 7.2). Several regions show extreme peaks and valleys of insertion frequency, which suggest "hot" and "cold" spots for T-DNA integration (Fig. 7.2). The density of insertion events was correlated with expressed rather than predicted gene density along each chromosome [49, 128, 157].

T-DNA insertions are found at low frequency in repeated DNA and transposable element (TE)-related sequences and at high frequency in gene-rich regions. Gene intervals with a strong bias toward the 5′ upstream and 3′ downstream regions of the genes contain 55–63 % of the T-DNA inserts [44, 49, 128, 157] (Table 7.4). Functional classification of the tagged genes showed a distribution similar to that for all genes in the rice chromosomes, so the T-DNA insertion is not biased toward a particular class of genes [4, 128]. The overall GC content at the insertion sites is close to that measured from the entire rice genome. The analysis of DNA sequence compositions around the T-DNA insertion sites revealed several prominent features, including elevated bendability from −200 to 200 bp relative to the insertion sites, an inverse relationship between the GC and TA skews, and reversed GC and TA skews in sequences upstream and downstream of the insertion sites, with both GC and TA skews equal to zero at the insertion sites [157].

3.2 *Tos17*

Sequence indexing of ~40,000 new *Tos17* insertions induced by tissue culture in cv. Nipponbare, in independent laboratories, with different tissue culture and FST production protocols, yielded remarkably consistent results [101, 123]. Like T-DNA inserts, new *Tos17* inserts are preferentially recovered from gene-dense regions over centromeric heterochromatin regions [101]. Interestingly, the distribution of the two elements over the rice chromosomes shows overlapping peaks of insertions (Fig. 7.2). However, *Tos17* inserts fall into gene intervals and preferably in introns and exons, at a higher frequency (75–85 %) than T-DNA inserts. This feature is favorable to inactivate gene transcription, although experience has shown that insertion of *Tos17* in intron sequences can be efficiently spliced out and thus has no influence on transcript abundance. *Tos17* also exhibits a clear preference for certain genes because the mean number of sequence-indexed allelic insertions in *Tos17* target genes is ~3 and can be up to >200 alleles in

Table 7.3 Distribution of the sequence-indexed inserts on the 12 rice chromosomes according to the nature of the mutagen

Chromosome	Length (Mb)	Nb of genes	Predicted gene density/Mb	*Ds* inserts	*Ds* density/Mb	*dSpm* inserts	*dSpm* density/Mb	T-DNA inserts	T-DNA density/Mb	*Tos17* inserts	*Tos17* density/Mb
Os01	43,27,0923	5,069	117.15	1,054	24.36	1,476	34.11	24,030	555.34	4,848	112.04
Os02	35,93,725	4,134	115.03	932	25.93	2,008	55.88	18,688	520.02	4,604	128.11
Os03	36,413,819	4,386	120.45	1,028	28.23	1,471	40.40	23,454	644.10	4,109	112.84
Os04	35,502,694	3,416	96.22	920	25.91	1,099	30.96	14,989	422.19	3,347	94.27
Os05	29,958,434	3,116	104.01	359	11.98	725	24.20	12,549	418.88	2,996	100.01
Os06	31,248,787	3,232	103.43	434	13.89	826	26.43	12,395	396.66	3,492	111.75
Os07	29,697,621	3,061	103.07	502	16.90	743	25.02	12,124	408.25	5,582	187.96
Os08	28,443,022	2,759	97.00	522	18.35	710	24.96	10,670	375.14	2,747	96.58
Os09	23,01,272	2,259	98.16	299	12.99	650	28.25	9,593	416.86	2,199	95.56
Os10	23,207,287	2,292	98.76	428	18.44	582	25.08	9,006	388.07	2,223	95.79
Os11	29,021,106	2,704	93.17	463	15.95	740	25.50	9,434	325.07	2,092	72.09
Os12	27,531,856	2,438	88.55	385	13.98	627	22.77	9,225	335.07	2,378	86.37
Total	373,245,519	38,866	104.13	7,326	19.63	11,657	31.23	166,157	445.17	40,617	108.82
r^2 gene/insert densities					0.64		0.72		0.94		0.42

r^2 shows correlations between chromosomal insertion mutagen densities and gene densities for each mutagen

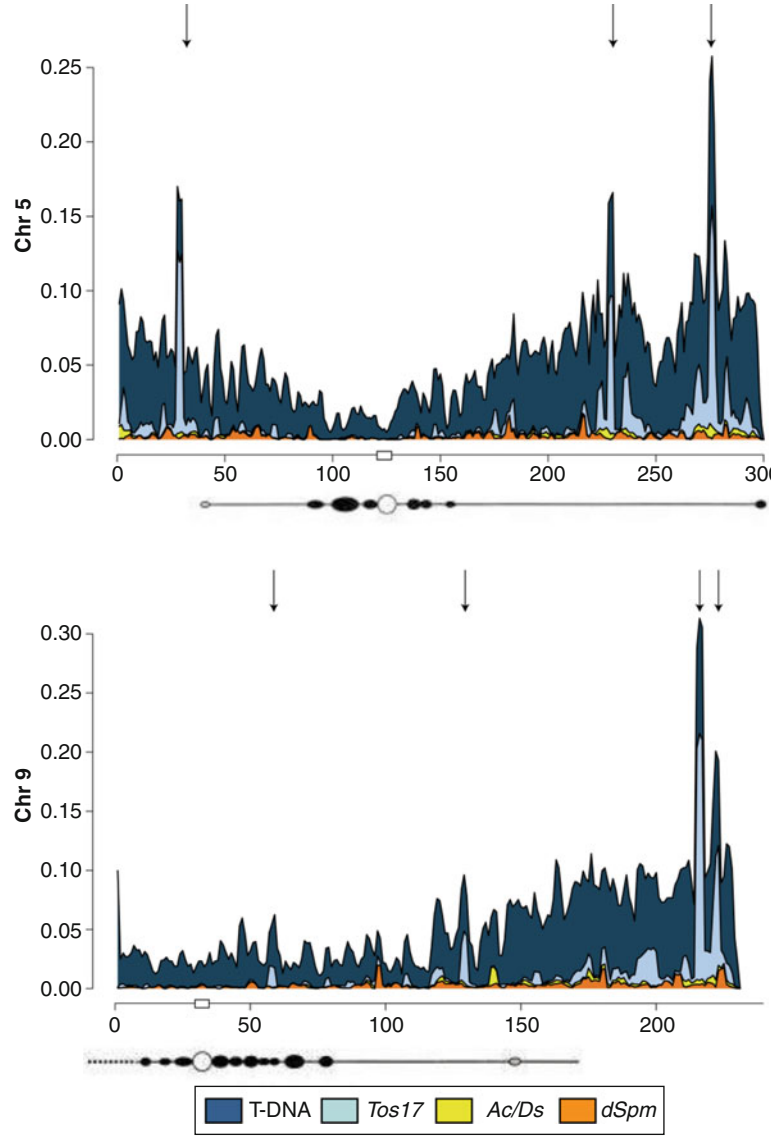

Fig. 7.2 Histograms of distribution of T-DNA, *Tos17*, *Ds*, and *dSpm* inserts along the rice chromosomes: examples are given for chromosomes 5 and 9. Position of the centromere on the pseudomolecule is indicated by an empty rectangle. FSTs corresponding to unique insertion positions were plotted at 200-kbp intervals by use of the joint MSU and RAP release v7 of the rice genome sequence. Like most rice chromosomes, chromosome 5 exhibits a higher frequency of inserts in subtelomeric regions than centromeric regions. Note that some peaks of insertions for *Tos17* and T-DNA inserts overlap at the same intervals (*arrows*). Chromosomes 4, 9, and 10 have short arms, which are known to be heterochromatic. The short arms (here illustrated with chromosome 9) exhibit a rather uniform and low frequency of insertion, whereas the long arms display a sharp increase of inserts in the subtelomeric region. Below the histograms are ideograms depicting the distribution of heterochromatin in the corresponding Nipponbare pachytene chromosome based on staining patterns with 4′,6-diamidino-2-phenylindole (DAPI) [13]. DAPI-bright regions are represented by *dark circles*. *Open circles* represent the location of the centromeres. The dotted line on the top of chromosome 9 represents the rDNA section. Although an exact correspondence between sequence intervals and DAPI-bright regions cannot be established, this representation provides a glimpse into the relationships between the chromatin status and amenability to integrate foreign DNA sequences in large chromosomal regions

Table 7.4 Distribution of the sequence-indexed inserts in genome compartments by nature of the mutagen and origin of the insertion mutant collection

Mutagen	Source	Mapped	Intergenic	%	Genic	%	Promoter	%	5′UTR	%	Exon	%	Intron	%	3′UTR	%
Ds	CSIRO	572	151	26.4	421	73.6	96	22.8	19	4.5	170	40.4	86	20.4	50	11.9
Ds	OSTID	1,301	379	29.1	922	70.9	203	22.0	28	3.0	352	38.2	206	22.3	133	14.4
Ds	PMBBRC	1,034	258	25.0	776	75.0	175	22.6	28	3.6	300	38.7	173	22.3	100	12.9
Ds	UCD	4,419	1,554	35.2	2,865	64.8	763	26.6	156	5.4	890	31.1	674	23.5	382	13.3
Dspm	UCD	11,657	6,144	52.7	5,513	47.3	1,576	28.6	117	2.1	1,337	24.3	1,139	20.7	1,344	24.4
T-DNA	OTL	24,256	11,104	45.8	13,152	54.2	4,403	33.5	608	4.6	2,482	18.9	3,423	26.0	2,236	17.0
T-DNA	POSTECH	100,447	32,133	32.0	63,404	63.1	19,615	30.9	3,398	5.4	13,660	21.5	17,110	27.0	9,621	15.2
T-DNA	RMD	21,239	8,555	40.3	12,684	59.7	4,016	31.7	617	4.9	2,562	20.2	3,372	26.6	2,117	16.7
T-DNA	ShIP	8,875	3,442	38.8	5,433	61.2	1,923	35.4	273	5.0	892	16.4	1,215	22.4	1,130	20.8
T-DNA	TRIM	11,340	4,305	38.0	7,035	62.0	2,423	34.4	505	7.2	1,565	22.2	1,562	22.2	980	13.9
Tos17	OTL	13,874	1,889	13.6	11,985	86.4	995	8.3	189	1.6	5,500	45.9	4,369	36.5	932	7.8
Tos17	RMD	8,913	4,040	45.3	4,873	54.7	755	15.5	115	2.4	1,577	32.4	1,926	39.5	500	1.3
Tos17	NIAS	17,830	2,455	13.8	15,375	86.2	1,615	10.5	277	1.8	6,463	42.0	5,593	36.4	1,427	9.3
		225,757	76,409		144,579		38,558	26.7	6,330	4.4	37,750	26.1	40,848	28.3	20,952	14.5

Table 7.5 Allelic insertions in each collection by the nature of the mutagen

Mutagen	Source	No. of rice genes with *x* inserts			Total genes interrupted	Insert/gene	
		1	2	3 or more		Average	Max
Ds	CSIRO	280	24	4	308	1.12	4
Ds	OSTID	471	49	14	534	1.17	7
Ds	PMBBRC	784	30	1	815	1.04	3
Ds	UCD	1,016	151	80	1,247	1.32	17
Dspm	UCD	1,945	388	188	2,521	1.35	14
T-DNA	OTL	5,642	954	238	6,834	1.22	11
T-DNA	POSTECH	10,405	5,565	5,615	21,585	2.03	40
T-DNA	RMD	5,096	1,139	437	6,672	1.33	13
T-DNA	ShIP	1,376	283	107	1,766	1.31	6
T-DNA	TRIM	4,126	528	99	4,753	1.17	22
Tos17	OTL	1,465	442	779	2,686	2.92	55
Tos17	RMD	1,635	330	246	2,211	1.61	37
Tos17	NIAS	2,065	644	1,087	3,796	2.87	74
Nb of rice genes	38,866	8,360	6,131	13,441	27,932		

27,932 Rice genes (71.9 % of the predicted non-transposable-element gene complement) have at least one insert disrupting their sequence (−1000ATG to +300 STOP codon interval)

the current NIAS and OTL collections [123], while the number of allelic insertions per T-DNA-tagged gene ranges from 1.2 to ~2 depending on collection size (Table 7.5). This feature is favorable for investigating allelic disruption in a given gene in the same collection source but implies that saturating the rice gene complement with only *Tos17* insertions may prove intractable. Analysis of insertion target sequences revealed a palindromic consensus sequence, ANGTT-TSD-AACNT, flanking the 5-bp TSD [101].

3.3 *Ds* and *dSpm*

Current sequence-indexed *Ds* and *dSpm* insertions result from a rather limited number of original T-DNA transposon launching pads that may create a bias toward positions linked to the T-DNA insertion sites. However, analysis of 7,000 and 11,000 *Ds* and *dSpm* insertions, respectively, allowed for establishing general trends for maize transposon behavior in the rice genome. *Ds* preferentially inserts into genic regions (64–75 %), whereas sequence-indexed *dSpm* inserts have a more balanced distribution among intergenic and genic regions that resembles that of T-DNA inserts (Table 7.4). The *Spm*/*dSpm* system generates more unlinked transpositions than does *Ac*/*Ds*, with lower intrachromosomal transposition rate (12 % vs. 24 %) and lower frequency of transposition in the T-DNA launching pad (3.4 % vs. 12 %) [68, 71]. Moreover *dSpm* seems less prone to "hot spots" of insertions than the *Ds* element. *Ds* preferentially inserts into promoter, intron and exon regions with frequencies that appear intermediary between those of T-DNA and *Tos17*. However, *dSpm* insertion frequencies in gene compartments are again closer to that for T-DNA. Because *Ds* and *dSpm* have different preferences for insertion in the rice genome, different genome coverage is likely to be achieved when using these elements [71].

3.4 *nDART1*

Analysis of a limited set of newly inserted sites of nDart1 elements revealed a predominant integration into single-copy regions. More than 60 % of the transposed elements were inserted into the −500/+500 predicted gene intervals. Two-thirds of the insertions resided in the 0.5-kb region upstream of ATG. These preliminary data suggest that nDart1 might be an interesting, somaclonal

variation-free complementary gene-tagging system for rice functional genomics [135]

4 Current Genome Coverage of Sequence-Indexed Insertions and Use in Reverse Genetics

Krysan and coworkers estimated that 180,000 and 280,000 inserts are needed for a 95 or 99 % chance of finding at least one insert in any *Arabidopsis* gene of average 2.8-kb length [70]. With 385,000 sequence-indexed inserts, including 325,000 T-DNA inserts, 12.2 % of TAIR9-annotated genes still lack insertions, and 8.2 % of the genes have only one allele [112]. Using a similar equation, Zhang and coworkers estimated that 365,380 inserts are needed to saturate the genome with a probability of 0.95. On the basis of the 45,441 FSTs released in databases and the genome annotation available in 2007, 14,287 of the 42,653 non-TE-related genes were found interrupted by at least one insert [157]. A reexamination of these figures 5 years later with the current genome coverage of unique insertion points (226,000) and the latest joint Rice Annotation Project (RAP) and MSU release v7.0 revealed that 144,000 inserts (64 %) fall in a gene interval spanning from −1,000 upstream of the ATG to +300 downstream of the STOP codon (Table 7.4). More than two-thirds (72 %) (27,932) of the 38,866 predicted genes are interrupted by at least one sequence-indexed insertion (Table 7.5). What could appear as a slower gain vs. effort progression compared to the 2007 figures is due to the asymptotic path to reach genome saturation. As an illustration, the 21,585 genes tagged by sequence-indexed T-DNA inserts in the large POSTECH collection harbors a mean of 2.03 allelic inserts, whereas the 6,834 genes tagged in the smaller OTL collection harbors a mean of 1.2 allelic inserts. Allelic insertions are desirable for several reasons. First, T-DNA FSTs have a variable confirmation rate (60–80 %) in rice [93], whereas the largest *Arabidopsis* T-DNA collections exhibit a 76 % confirmation rate [112]. Second, integration of a mutagen in a gene interval does not always create a knock out, notably when it inserts into an intron, a 3′UTR sequence or a position far from the ATG. Current sequence-indexed insertions interrupt an annotated promoter, 5′UTR, exon, intron, and 3′UTR sequences with 26.7 %, 4.4 %, 26.1 %, 28.3 %, and 14.5 % frequencies, respectively. Third, having two confirmed independent allelic insertions creating knockouts with convergent phenotypes avoids a tedious complementation step in gene function establishment. So far, one-third (13,441) of the rice genes are interrupted by three or more insertions. The insertion lines currently available for distribution in rice likely contain a sufficient number of insertions of known mutagens. For instance, the 400,000 T-DNA insertion lines contain a mean of 1.4 integration loci (i.e., 560,000 putative inserts). Therefore, we need to pursue the FST generation effort. As a complement, TILLING services can be used to search for lesions in genes without inserts and alleles in genes with only one verified insertion-mediated knockout.

Finding a mutation in a GOI was first accomplished by PCR amplification in 2D or 3D pools of mutant DNA with use of gene- and mutagen-specific primers followed by deconvoluting the identified pool into individual single lines. This method has been used with success to find T-DNA insertions in 12 MADS box genes, which notably control developmental processes. A population of 21,049 lines was resolved in DNA pools of 500–1,000 lines that were adequate for screening a T-DNA insertion within a 2-kb region, thus allowing the identification of five insertions in four different genes in this limited mutant population [79]. The same method has been used to identify *Tos17* alleles in T-DNA lines.

The systematic sequencing of chromosomal regions flanking insertion points by PCR-based methods has facilitated direct in silico access to mutant seed stocks through dedicated and user-friendly genome navigators such as Rice GE and OrygenesDB. The functions of a large range of genes involved in cell and developmental processes and in plant response to biotic and abiotic environments have been unraveled by use of mutant resources and in silico reverse genetics (Table 7.6).

Table 7.6 Main published reports of successful implementation of forward and reverse genetics strategies used with insertion mutants from international collections in rice

Class	Trait	Gene	Mutagen	Collection	Strategy	Remarks	References
Abiotic stress response	Water homeostasis	*COW1*	T-DNA+*Tos17*	POSTECH	Reverse		[147]
	Viviparity	*OsABA1*	*Tos17*	NIAS	Forward	Screen of viviparous mutants	[3]
	ABA signaling	*OsABF2*	T-DNA	POSTECH	Reverse		[42]
	Calcium signaling	*OsCIPK31*	Ds	Gyeongsan Natl. Univ.	Reverse		[122]
	Chloride channels	*OsCLC1*, *OsCLC3*	*Tos17*	NIAS	Reverse		[105]
	Low temperature responsive	*OsDMKT1*	T-DNA	POSTECH	Forward		[82]
	ABA and xanthophyll synthesis	*OsDSM2*	T-DNA	RMD	Forward	Screen for drought tolerance	[22]
	Glutamate receptor in RAM	*OsGLR3.1*	T-DNA	RMD	Forward		[87]
	Metal homeostasis	*OsHMA9*	T-DNA	POSTECH	Reverse		[81]
	Osmolyte synthesis	*OsITPK2*	T-DNA	RMD	Forward	Screen for drought tolerance	[21]
	Oxidative stress	*OsPUB15*	T-DNA	POSTECH	Reverse		[116]
	Low temperature responsive	*OsRLK1*	T-DNA	POSTECH	Forward	Gene trapping	[77, 82]
	Zinc transport	*OsZIP5*	T-DNA	POSTECH	Reverse		[76]
	Zinc transport	*OsZIP8*	T-DNA	POSTECH	Reverse		[80]
Biotic stress response	Pathogen resistance	*BWMK1*	T-DNA	OTL	Reverse	Constitution of a resource	[18]
	Pathogen resistance	*CEBOP*	T-DNA	OTL	Reverse	Constitution of a resource	[18]
	Pathogen resistance	*NH1*	T-DNA	OTL	Reverse	Constitution of a resource	[18]
	Symbiosis	*OsCASTOR*	T-DNA	POSTECH	Reverse		[11]
	Pathogen resistance	*OsEDS5*	T-DNA	OTL	Reverse	Constitution of a resource	[18]
	Symbiosis	*OsPOLLUX*	*Tos17*	NIAS	Reverse		[11]
	Calcium channel	*OsTPC1*	*Tos17*	NIAS	Reverse		[72]
	Pathogen resistance	*OsWRKY22*	T-DNA	OTL	Reverse		[1]
	Pathogen resistance	*OsWRKY28*	T-DNA	OTL	Reverse	Constitution of a resource	[18]
	Pathogen resistance	*Pi21*	T-DNA	OTL	Reverse	Constitution of a resource	[18]
	Pathogen resistance	*rTGA2.1*	T-DNA	OTL	Reverse	Constitution of a resource	[18]
	Pathogen resistance	*SPL18*	T-DNA	NIAS	Forward	Activation tagged/lesion mimic	[103]
	Pathogen resistance	*SPL7*	T-DNA	OTL	Reverse	Constitution of a resource	[18]

Development	Spikelet formation	*BFL1*	Ds	CSIRO	Forward	Screen for inflorescence defects	[160]
	Glume formation	*DH1*	T-DNA	RMD	Forward	Screen for inflorescence defects	[84]
	Endosperm development	*EMF2b*	T-DNA	POSTECH + OTL	Reverse		[94]
	Endosperm development	*FIE1*	T-DNA	POSTECH + OTL	Reverse		[94]
	Regulation of meristem size	*FON1*	T-DNA + *Tos17*	POSTECH	Reverse	*Tos17* allele in T-DNA library	[102]
	Ligule and lamina joint development	*LG1*	T-DNA	POSTECH	Forward	3 Alleles	[75]
	Brassinosteroid regulator	*OdGSK1*	T-DNA	POSTECH	Forward	Screen for abiotic stress	[66]
	Dwarfism	*OsCPS1*	*Tos17*	NIAS	Reverse	Gibberelin synthesis mutants	[127]
	Root hair development, cellulose synthesis	*OsCSLD1*	Ds	Gyeongsan Natl. Univ.	Reverse		[64]
	Root and shoot architecture	*OsGA2ox*	T-DNA	TRIM	Forward	Activation tagging	[92]
	Internode elongation	*OsH15*	*Tos17*	NIAS	Reverse		[130]
	Leaf development	*OSH6*	Ds	Gyeongsan Natl. Univ.	Reverse		[118]
	Leaf angle	*Osila1*	T-DNA	RMD	Forward		[107]
	Dwarfism	*OsKAO*	*Tos17*	NIAS	Reverse	Gibberelin synthesis mutants	[127]
	Dwarfism	*OsKO2*	*Tos17*	NIAS	Reverse	Gibberelin synthesis mutants	[127]
	Dwarfism	*OsKS1*	*Tos17*	NIAS	Reverse	Gibberelin synthesis mutants	[127]
	Shoot elongation	*OsKS1*	Ds	CSIRO	Forward	Screen for dwarf mutants	[96]
	Photomorphogenesis	*OsPhyA*	*Tos17*	NIAS	Reverse		[137]
	Photomorphogenesis	*OsPhyC*	*Tos17*	NIAS	Reverse		[136]
	Brassinosteroid	*OsRAVL1*	Ds	Gyeongsan Natl. Univ.	Reverse		[47]
	Leaf bulliform cell development	*OsRoc5*	T-DNA	RMD	Forward	Screen for leaf rolling	[162]
	Dwarfism, erect leaves	*XIAO*	T-DNA	RMD	Forward	Screen of dwarf mutants	[56]
Cell wall	Cellulose content	*OsCes4*	*Tos17*	NIAS	Forward	Screen for brittle culm mutants	[138]
	Cellulose content	*OsCes7*	*Tos17*	NIAS	Forward	Screen for brittle culm mutants	[138]
	Cellulose content	*OsCes9*	*Tos17*	NIAS	Forward	Screen for brittle culm mutants	[138]
	Cellulose content	*OsBC1L4*	T-DNA	RMD	Forward	Screen for dwarf mutants	[17]
	Cuticle formation	*OsWDL1*	T-DNA	POSTECH	Forward	Screen for stunted mutant	[115]
	Lignin synthesis	*OsFC1*	T-DNA	RMD	Forward	Screen for culm strength defect	[90]

(continued)

Table 7.6 (continued)

Class	Trait	Gene	Mutagen	Collection	Strategy	Remarks	References
Photosynthesis	Chlorophyll synthesis	*OsCAO1*	T-DNA + *Tos17*	POSTECH + NIAS	Reverse		[78]
	Chloroplast galactolipid synthesis	*OsLIGE*	T-DNA	RMD	Forward	Screen for stunted mutant	[85]
	Photosynthetic integrity	*Osphot1*	T-DNA + *Tos17*	POSTECH	Reverse		[32]
Respiration	Mitochondrial RNA editing	*OsOGR1*	T-DNA	POSTECH	Reverse		[65]
DNA repair and maintenance	DNA repair	*OsKu70*	T-DNA	POSTECH	Reverse		[40]
	DNA replication and repair	*OsRPA1*	T-DNA	RMD	Reverse		[10]
	Telomere length and architecture	*RTPB1*	T-DNA	POSTECH	Reverse		[41]
Flowering	Flowering transition and ethylene	*OsETR2*	T-DNA	RMD	Reverse		[151]
	Floral organ identity	*OsMADS1*	*Tos17*	NIAS	Reverse		[2]
	Floral organ identity	*OsMADS3*	T-DNA + *Tos17*	POSTECH + NIAS	Reverse		[152]
	Flowering transition	*RID1*	T-DNA	RMD	Forward		[149]
	Flowering time repressor	*OsCOL4*	T-DNA	POSTECH	Reverse		[83]
Meiosis	Bivalent formation at meiosis	*OsPAIR1*	*Tos17*	NIAS	Forward		[111]
	Bivalent formation at meiosis	*OsPAIR2*	*Tos17*	NIAS	Forward		[110]
	Bivalent formation at meiosis	*OsPAIR3*	T-DNA	RMD	Reverse		[156]

Pollen/anther development and pollen germination	Anther dehiscence	*AID1*	Ds	CSIRO	Forward	Screen for inflorescence defects	[161]
	Pollen development	*OsAPI5*	T-DNA	RMD	Forward		[88]
	Pollen development	*OsCP1*	T-DNA	POSTECH	Forward		[77]
	Tapetum development	*OsDMT1*	T-DNA	POSTECH	Reverse		[155]
	Nuclear import, pollen tube elongation	*OsImpβ1*	T-DNA	POSTECH	Forward	Gene trapping	[35]
	Regulation of sporocyte number	*OsMSP1*	*Tos17*	NIAS	Forward	Screen for sterility	[109]
	Pollen development and germination	*OsNOP1*	Ds	Temasek	Forward	Deletion	[55]
	Pollen development	*OsRIP1*	T-DNA	POSTECH	Forward	Gene trapping	[36]
	Tapetum development	*OsUDT1*	T-DNA + *Tos17*	POSTECH	Forward	Gene trapping-aided, *Tos17* allele	[59]
	SUMO E2 ligase anther dehiscence	*SIZ1*	T-DNA	TRIM	Forward		[139]
Seed development, maturation, and germination	Viviparity	*OsABA1*	*Tos17*	NIAS	Forward	Screen of viviparous mutants	[3]
	Seed maturation	*OsALDH7*	T-DNA	POSTECH	Reverse		[131]
	Seed abcission	*OsCPL1*	T-DNA	POSTECH	Reverse	Point mutant T-DNA alleles	[51]
	Seed germination	*OsDSG1*	T-DNA	POSTECH	Reverse		[114]
	Control of α-amylase expression in aleurone cells	*OsGAMYB*	*Tos17*	NIAS	Reverse	4 Alleles	[61]
	Growth and grain filling	*OsGS1;1*	*Tos17*	NIAS	Reverse		[134]
	Starch biosynthesis regulation	*OsRSR1*	T-DNA	SHiP	Reverse		[25]
	Grain endosperm starch	*OsSSI*	*Tos17*	NIAS	Reverse	Chemical mutant allele	[30]
	Grain endosperm starch	*OsSSIII*	*Tos17*	NIAS	Reverse		[31]
	Grain endosperm starch	*PUL*	*Tos17*	NIAS	Reverse		[29]
	Folate synthesis	*FPGS*	T-DNA	POSTECH	Reverse		[5]

5 Use of Insertion Mutant Resources in Forward Genetics

5.1 Spectrum of Variation and Discovered Genes

A large range of phenotypic variations has been observed in the rice mutant resources of T-DNA [43, 93, 158], *Tos17* [99], and *Ds* [54, 113] in field evaluation under agronomic conditions. The most affected categories of traits are seed germination and plant growth, culm and leaf color (including spotted, lesion mimic) and shape, tillering, heading date, fertility, and seed, spikelet, and panicle morphology. The frequencies observed in large *Tos17* and T-DNA mutant collections are in Table 7.7. Insertion lines have also been subjected to specific screens such as for developmental traits (dwarfism), fertility, grain filling, development and viviparity, response to artificial inoculation of pathogens or field drought (e.g., [26, 93]). Whether a lesion in a given sequence co-segregates with an alteration of the phenotype, a link can be established between a phenotype and an inactivated gene by the "forward genetics" approach. The difficulty resides in the fact that rice insertion mutagenesis mainly relies on plant regeneration from tissue culture, which is known to generate somaclonal variation, a sum of genetic and epigenetic changes that has been well documented in rice. However, somaclonal variation might not be the only cause of absence of linkage, because low frequencies of tagging are also observed in *Arabidopsis* T-DNA insertion lines generated by the floral dip method.

In rice *Tos17* and T-DNA mutant populations, 3–5 % of the observed alterations are actually tagged by the mutagen [93, 109]. This frequency is likely to vary by the trait investigated and the robustness of the phenotypic screen. Despite these limitations, an increasing number of genes, notably those involved in hormone synthesis, cell wall synthesis, leaf anatomy, pollen/anther development, spikelet formation, and response to abiotic stresses, have been isolated by a forward genetics approach in rice *Tos17*, *Ds*, or T-DNA mutants (Table 7.6).

Hormones: Two mutants impaired in abscisic acid (ABA) biosynthesis have been identified by forward genetics: *Osaba1*, a strong viviparous *Tos17* mutant with a wilt phenotype [3], and *dsm2*, a drought-hypersensitive T-DNA mutant, with insertions in a homolog of zeaxanthin epoxidase and a putative β-carotene hydroxylase, respectively, exhibit impaired biosynthesis of zeaxanthin, a carotenoid precursor of ABA [22]. A severe dwarf transposon (*Ds*) insertion mutant was found altered in a putative ent-kaurene synthase gene that encodes the enzyme catalyzing the second step of the gibberellin (GA) synthesis [96]. XIAO was recently identified by the characterization of a T-DNA mutant exhibiting dwarfism and erect leaves, with reduced seed setting. XIAO also exhibited an enhanced tissue-specific brassinosteroid (BR) response and greatly reduced BR content at the whole-plant level. XIAO was predicted to encode a leucine-rich-repeat (LRR) kinase that would regulate BR signaling and cell division [56].

Secondary cell wall synthesis: Three distinct brittle culm mutations caused by *Tos17* led to the identification of CesA (cellulose synthase catalytic subunit) genes, OsCesA4, OsCesA7, and OsCesA9, participating in the cellulose-synthesizing complex involved in the synthesis of the secondary cell wall [138]. Another mutation altering cellulose synthesis, identified in the T-DNA mutant *Oryza sativa brittle culm 1 like 4* (*Osbc1l4*), affects a COBRA-like protein that exhibits typical structural features of a glycosylphosphatidylinositol-anchor protein [17]. Another T-DNA mutant, *wilted dwarf and lethal 1* (*wdl1*), exhibiting dwarfism and early death, is altered in a gene encoding a protein belonging to the SGNH subfamily within the GDSL lipase superfamily. *Wdl1* leaves have a disorganized crystal wax layer, which suggests that WDL1 is involved in cutin organization, affecting depolymerizable components [115]. Finally, the function of a gene playing an important role in the biosynthesis of lignin and control of culm strength in rice, *FLEXIBLE CULM 1* (FC1), was identified through a T-DNA mutant screen. *FC1* encodes a cinnamyl-alcohol dehydrogenase mainly expressed in the sclerenchyma cells of the secondary cell wall and vascular

Table 7.7 Correspondence between categories and subcategories of altered traits from field evaluations of Nipponbare NIAS *Tos17* lines [99], Tainung 67 TRIM T-DNA activation tagging lines [14] and Nipponbare T-DNA lines [93]

From Miyao et al. [99]	Number of traits: 53		Total lines 50,000	From Chern et al. [14]	Number of traits: 61		Total lines 22, 665	From Lorieux et al. [93]	Number of traits: 71		Total lines 27,832
Category	Subcategory	# Lines	Freq	Category	Subcategory	# Lines	Freq	Category	Subcategory	# Lines	Freq
1 Germination	Low germination rate	3,489	6.98	Growth	Germination rate	1	0.00	Germination	Germination rate	6,108	21.95
2 Growth	Lethal	1,630	3.26		Lethal	139	0.61	Growth	Lethal	560	2.01
	Abnormal shoot	1,787	3.57		Abnormal plants	10	0.04		Abnormal plants	210	0.75
	Weak	1,607	3.21		Weak	105	0.46		Weak	286	1.03
3 Leaf color	Albino	1,407	2.81	Leaf color	Albino	13	0.06	Leaf color	Albino	186	0.67
	Yellow	810	1.62		Yellow leaf	21	0.09		Yellow leaf	653	2.35
	Dark green	1,065	2.13		Dark green leaf	931	4.11		Dark green leaf	29	0.10
	Pale green	1,732	3.46		Pale green leaf	286	1.26		Pale green leaf	19	0.07
	Virescent	834	1.67		Bluish green leaf	69	0.30		Virescent	4	0.01
	Stripe	397	0.79		Stripe	46	0.20		Stripe	254	0.91
	Zebra	109	0.22		Zebra	7	0.03		Zebra	17	0.06
	Others	85	0.17		Others	159	0.70		Stay green	2	0.01
									Others	3	0.01
4 Leaf shape	Wide leaf	143	0.29	Leaf morphology	Wide leaf	186	0.82	Leaf morphology	Wide leaf	92	0.33
	Narrow leaf	1,379	2.76		Narrow leaf	1,119	4.94		Narrow leaf	48	0.17
	Long leaf	30	0.06		Long leaf	370	1.63		Long leaf	87	0.31
	Short leaf	40	0.08		Short leaf	540	2.38		Short leaf	32	0.11
	Drooping leaf	227	0.45		Drooping leaf	66	0.29		Drooping leaf	32	0.11
	Rolled leaf	343	0.69		Rolled leaf	550	2.43		Rolled leaf	88	0.32
	Spiral leaf	111	0.22		Spiral leaf	323	1.43		Spiral leaf	18	0.06
	Brittle leaf/culm	124	0.25		Brittle leaf/culm	14	0.06		Brittle leaf/culm	14	0.05
	Abnormal lamina joint angle	96	0.19		Thin lamina joint	5	0.02		Horizontal leaf	330	1.19
	Withering	888	1.78		Withering	160	0.71		Erect leaf	69	0.25
	Others	346	0.69		Others	127	0.56		Others	64	0.23

(continued)

Table 7.7 (continued)

From Miyao et al. [99]	Number of traits: 53		Total lines 50,000	From Chern et al. [14]	Number of traits: 61		Total lines 22, 665	From Lorieux et al. [93]	Number of traits: 71		Total lines 27,832
Category	Subcategory	# Lines	Freq	Category	Subcategory	# Lines	Freq	Category	Subcategory	# Lines	Freq
5 Culm shape	Semi-dwarf	3,670	7.34	Plant morphology	Semidwarf	872	3.85	Plant morphology	Semidwarf	847	3.04
	Dwarf	5,722	11.44		Dwarf	1,242	5.48		Dwarf	250	0.90
	Severely dwarf	1,376	2.75		Extremely dwarf	111	0.49		Long culm	136	0.49
	Long culm	679	1.36		Long culm	24	0.11		Erect	5	0.02
	Fine culm	18	0.04		Erect	194	0.86		Spread-out	104	0.37
	Thick culm	63	0.13		Spread-out	413	1.82		Thin culm	21	0.08
	Lazy	376	0.75		Thin culm	873	3.85		Thick culm	24	0.09
	Others	8	0.02		Thick culm	21	0.09		Lazy	28	0.10
					Lazy	1	0.00		Compact	90	0.32
									Culm branching	7	0.03
6 Spotted leaf/ lesion mimic	Spotted leaf/ lesion mimic	1,102	2.20	Mimic response	Lesion mimic	258	1.14	Mimic response	Lesion mimic	273	0.98
7 Tillering	High tillering	111	0.22	Tiller	High tiller position	8	0.04	Tiller	High tillering	79	0.28
	Low tillering	2,837	5.67		Low tiller position	0	0.00		Low tillering	167	0.60
					Monoculm	74	0.33		Monoculm	35	0.13
					Few panicle	352	1.55				
					Many panicle	1,092	4.82				
8 Heading date	Early heading	1,797	3.59	Heading data	Early heading	14	0.06	Heading data	Early heading	0	0.00
	Late heading	1,244	2.49		Late heading	195	0.86		Late heading	401	1.44
	Non-heading	96	0.19		No heading	140	0.62		No heading	0	0.00
9 Spikelet	Abnormal hull	508	1.02	Flower	Abnormal hull	38	0.17	Flower	Abnormal hull	223	0.80
	Abnormal floral organ	211	0.42		Abnormal floral orgar	1	0.00		Abnormal floral organ	0	0.00
					With awn	36	0.16		Awned	106	0.38
					Abnormal hull	9	0.04		Abnormal hull color	52	0.19
					Abnormal hull color	91	0.40		Others	3	0.01

Category	Subcategory	# Lines	Freq	Category	Subcategory	# Lines	Freq	Category	Subcategory	# Lines	Freq
10 Panicle	Long panicle	29	0.06	Panicle morphology	Long panicle	35	0.15	Panicle morphology	Long panicle	4	0.01
	Short panicle	752	1.50		Short panicle	939	4.14		Short panicle	4	0.01
	Lax panicle	104	0.21		Sparse panicle	270	1.19		Sparse panicle	0	0.00
	Dense panicle	264	0.53		Dense panicle	189	0.83		Dense panicle	28	0.10
	Viviparous	1,105	2.21		Viviparity	0	0.00		Shattering	0	0.00
	Shattering	6	0.01		Shattering	5	0.02		Neck leaf	0	0.00
	Neck leaf	171	0.34		Neck leaf	36	0.16		Abnormal panicle shape	120	0.43
	Abnormal panicle shape	433	0.87		Abnormal panicle shape	178	0.79		Incomplete exertion	27	0.10
					Others	173	0.76		Few spikelets	40	0.14
									Terminal spikelet	8	0.03
									Others	49	0.18
11 Sterility	Sterile	3,825	7.65	Seed fertility	Sterile	571	2.52	Seed fertility	Sterile	1,311	4.71
	Low fertility	12,542	25.08		Low fertility	403	1.78				
12 Seed	Large grain	195	0.39	Seed fertility	Large grain	29	0.13	Seed morphology	Large grain	0	0.00
	Small grain	427	0.85		Small grain	1,063	4.69		Small grain	71	0.26
	Slender grain	101	0.20		Slender grain	29	0.13		Slender grain	0	0.00
	Others	3,469	6.94		Others	64	0.28		Others	0	0.00
								Root system	Weak root	22	0.08

Reproduced from [93]

bundle region. The *fc1* mutant exhibits, among other altered traits, a flexible culm, because of reduced cell wall thickness and decreased lignin [90].

Pollen and anther development: The *Tos17 multiple sporocyte 1* (*msp1*) mutant has an excessive number of both male and female sporocytes, disorganized anther wall layers and absence of tapetum, which results in complete male sterility. *MSP1* encodes an LRR receptor-like protein kinase (LRR-RLK) that plays crucial roles in restricting the number of cells entering male and female sporogenesis and initiating anther wall formation in rice [109]. A 65-kb deletion related to a *Ds* insertion into the *Oryza sativa no pollen* (*Osnop*) gene led to male sterility. *OsNOP* plays a role in male gametophyte development and likely encodes a C2-GRAM-domain-containing protein [55]. Use of *Ds* to interrupt the gene sequence of *ANTHER INDEHISCENCE1* (AID1), encoding a single MYB domain protein, triggers partial to complete spikelet sterility. The MYB domain of AID1 is closely related to that of the telomere-binding proteins of human, mouse, and Arabidopsis [161]. The integration of T-DNA in the 15th exon of a rice SUMO E3 ligase gene, SIZ1, led to anther dehiscence of a TRIM mutant [139].

Leaf anatomy and morphology: Several interesting mutants exhibiting leaf anatomical defects have been identified. They include the *outcurved leaf1* (*ucl1*) mutant exhibiting abaxial leaf rolling owing to a T-DNA insertion in the *RICE OUTERMOST CELL-SPECIFIC GENE5* (*ROC5*), an ortholog of the *Arabidopsis* homeodomain leucine zipper class IV gene GLABRA2. The mutation caused an increase in bulliform cell number and size, which indicates that *ROC5* negatively regulates bulliform cell fate and development [162]. The mutant *increased leaf angle 1* (*ila1*) resulted from a T-DNA insertion in a Raf-like MAPKKK with Ser/Thr kinase activity that interacts with a nuclear protein family and regulates mechanical tissue formation in the lamina joint of rice. The phenotype is caused by abnormal vascular bundle formation and cell wall composition in the leaf lamina joint [107]. The rice *liguleles 1s* (lg1) mutant shows complete loss of the auricle, ligule, and laminar joint resulting from a T-DNA insertion in a gene encoding a protein that contains a SQUAMOSA promoter binding protein domain, which is highly homologous to the maize *LIGULELESS1* (*LG1*) gene [75, 81].

Spikelet development: *Branched floretless 1* (*Bfl1*), a *Ds*-tagged rice mutant defective in the transition from spikelet meristem to floret meristem, fails to develop glumes and florets. Instead, axillary meristems in the bract-like structure produce sequential alternate branching, which results in coral-shaped features of the branches in the developing panicle. BFL1 is probably a rice ortholog of the maize ERF (EREBP/AP2) transcription factor gene BD1. Because of similarities in mutant phenotypes, *bfl1 i*s likely an allele of the previously reported frizzy panicle locus [160]. Another gene involved in glume formation identified by T-DNA tagging is an LOB domain-like protein DEGENERATED HULL1 (DH1), the disruption of which leads to degenerated hulls and naked pistils and stamens [84].

Abiotic stress response and photosynthesis: A *drought- and salt-hypersensitive mutant* (*dsm3*) is caused by a T-DNA insertion in a gene encoding a putative inositol 1,3,4-trisphosphate 5/6-kinase previously named OsITPK2, with unknown function. The mutant exhibits a deficit in osmolytes such as proline and soluble sugar under drought stress, as well as other morphological alterations [21]. Finally, a stunted-growth mutant with decreased carbon assimilate and yield production, *photoassimilate defective1* (*phd1*), resulted from a T-DNA insertion in a rice plastidial-nucleotide sugar epimerase involved in galactolipid biosynthesis [85, 86, 88]. PHD1 overexpression increased photosynthetic efficiency, biomass, and grain production, which suggests that it plays an important role in supplying sufficient galactolipids to thylakoid membranes for proper chloroplast biogenesis and photosynthetic activity.

5.2 Genes Discovered by Trapping and Activation Tagging

As mentioned above, engineered mutagens (T-DNA and *Ds* elements) for rice insertion

mutagenesis are equipped with a gene trap (GT) or an enhancer trap (ET) and/or an activation tag (AT) (Table 7.1 and Fig. 7.1 for details). Because these are dominant markers, reporter gene expression and AT-induced phenotypic alterations can be screened in primary regenerants. In rice, frequencies of gene detection based on trapping-mediated reporter gene activity range from 1.6 % to 8 % and 20 % to 30 % with GT and ET lines, respectively. Both the β-glucuronidase (GUSA) and GFP genes have been used as reporters in ET and GT constructs. Bidirectional gene traps involving the two reporter genes adjacent to each border of the T-DNA have allowed for comparing the sensitivity of the two systems [23, 126]. In these insertions, a lower frequency of detection with GFP than GUSA was attributed to sensitivity or the position of the GFP gene at the less-conserved left border of the T-DNA [126]. *Ds* elements were also effectively used to carry GT cassettes: 12 % of more than 15,000 lines examined expressed the GUS reporter gene during the early-seedling stage [113]. In this study, GUS was expressed in root hairs and crown root initials at estimated frequencies of 0.78 % and 0.34 %, respectively.

Ryu and coworkers established the frequency of effective GT-mediated detection of nearby genes and confirmed gene trapping in 19 of 25 tested GFP-positive lines by inverse PCR and isolation of rice gene sequences flanking the GFP trap [126]. Similarly, Yang et al. [154] assayed T1 seeds of 9,120 independent ET lines harboring a BOGUS::GFP N-terminal fusion. To evaluate the effectiveness of enhancer trapping, the authors selected 58 candidate promoters predicted from upstream flanking sequences. Of ten promoters (randomly amplified T-DNA FSTs) inserted upstream of the GUSA reporter gene, six exhibited consistent expression patterns with those of the original ET lines when reintroduced into rice by transformation [119].

The effectiveness of GT-mediated detection for discovery of novel genes has been demonstrated in examining conditionally regulated expression of the reporter gene in response to low temperatures [77, 82] or salt stress [66]. GT insertions characterized further at the molecular level allowed for identifying *OsRLK1*, an LRR-RLK gene inducible by cold and salt stress; *OsDMKT1*, a putative demethyl mena quinine methyl transferase induced under low temperatures; and *Oryza sativa* glycogen synthase kinase 3-like gene 1 (*OsGSK1*), a member of the plant GSK3/SHAGGY-like protein kinase genes and an orthologue of *Arabidopsis* BR-insensitive 2, *AtSK21*. The GUS staining pattern fully mirrored the sites of expression and the responsiveness of the trapped genes.

The other class of genes identified by GT are those expressed in floral organs. GUS histochemical assays of spikelets of 14,000 T-DNA GT lines identified 270 lines with GUS activity in anthers, of which 15 also exhibited male sterility co-segregating with the GUS pattern in progeny plants [60]. The mutant altered in undeveloped tapetum 1 (*udt1*) was subsequently isolated [59] UDT1 is a nuclear protein, and its transcripts are most abundant during early anther development. The cysteine protease *OsCP1* gene was isolated from a GT insert. The *OsCP1* promoter is highly active in loculi and tapetum of rice anthers and in developing pollen. Knockout mutants showed significant defects in pollen development and reduced height and seed formation. Two other genes implicated in pollen development and germination have been identified by GT insertion and histochemical assays of mutant anthers: rice Importin β1 (OsImpβ1) [35] and rice immature pollen 1 (Rip1) [36]. OsImpβ1 is accumulated in pollen and ovaries, where it interacts with nuclear transport factors and mediates the import of nuclear proteins. The mutant pollen matured normally, but pollen tube elongation was hampered in the mutant grains, which indicates that OsImpβ1 is specifically required in the process [35]. *RIP1* encodes a protein with significant homology to a group of proteins containing five WD40 repeat sequences and is expressed at the late stages of pollen development. *rip1* homozygous plants were not found in progenies of the mutant, whereas backcrossing showed the *rip1* mutant allele not paternally inherited. *Rip1* pollen exhibited delayed formation of starch granules and intine layer, and TEM observations identified ultrastructural alterations in mitochondria, Golgi

apparatus, lipid bodies, plastids, and endoplasmic reticulum. Thus, *RIP1* plays important cellular and developmental roles during the late stage of pollen formation.

Activation tagging (AT) is an alternative method to isolate genes with use of inserts carrying strong activating sequences that can quantitatively modify the transcription of genes adjacent to insertion sites while still retaining their original expression pattern (Fig. 7.1). To date, more than 160,000 T-DNA insertion lines harboring this system have been generated in the POSTECH and TRIM collections [44, 49, 50]. The possibility of randomly enhancing gene expression through T-DNA and *Ds*-mediated AT has been demonstrated in rice. Four of ten randomly chosen candidate lines exhibited enhanced expression of genes separated by a distance of 1.5–4.3 kb from the T-DNA enhancer elements while still maintaining their original expression pattern [50]. Similar results have been obtained through *Ds*-mediated AT [125].

A lesion mimic mutant, *Spotted leaf 18* (*Spl18*), was isolated from 13,000 activation-tagging lines [103]. The activated gene, situated 500 bp downstream from the T-DNA AT, *OsAT1*, showed sequence similarity to an acyltransferase with expression induced by hypersensitive reaction in tobacco. *Spl18* exhibited an enhanced resistance to blast disease. The gene was later overexpressed under the control of the 35S promoter and exhibited a phenotype similar to that with multiple pathogen resistance. Another gene identified through AT is a *GIBBERELIN 2 OXIDASE* (*GA2ox*). GA2oxs regulate plant growth by inactivating endogenous bioactive GAs. The GA2ox gene-activated mutant exhibited severe dwarfism, and the normal-height phenotype was restored with GA treatment, so GA2ox is one of the key enzymes regulating the endogenous level of GA [44]. GA2ox-overexpressing plants exhibited early and increased tillering and adventitious root growth. Thus, improvements in plant architecture, such as semi-dwarfism, increased root systems and higher tiller numbers, could be induced by overexpression of wild-type or modified GA2ox [92].

6 Transactivation with the GAL4-Enhancer Trapping System

A modification of the classical enhancer trap is the GAL4:GFP or GAL4:GUSA enhancer trapping system that was first successfully used in *Drosophila* [8, 121], then *Arabidopsis* [7, 62] (Fig. 7.1 for illustration and explanation). After identification of a particular enhancer trap line with interesting GUSA or GFP expression, a second construct can be introduced with genes of interest fused to UAS elements, which are expressed only in tissues and cell types expressing GUS or GFP. The Gal4:VP16/UAS-GFP and Gal4:VP16/UAS-GUSA ET system have been successfully incorporated into OTL and RMD T-DNA collections of rice insertion lines [58, 148, 154].

The enhancer trapping frequency with the GAL4 system used in rice T-DNA insertion lines ranged from 29 % with GFP [58] to 60 % to 70 % with GUS [148, 154]. As mentioned above, the GAL4-based system is unique in that it can be used as a tool to transactivate any transgene of interest fused to UAS elements. The transactivation was first exemplified by introducing a GUSA reporter gene fused to UAS elements in Gal4:VP16/UAS-GFP ET lines [58]. The efficiency of the GAL4-mediated transactivation system in rice was later expanded to target genes of interest [91, 124]. Liang and coworkers obtained hybrid plants by crossing target lines of ten putative transcription factor genes from rice with six "pattern" lines of the RMD library exhibiting expression in anther, stigma, palea, lemma, and leaves. Various phenotypic changes such as delayed flowering, multiple pistils, dwarfism, narrow and droopy leaves, reduced tillers, growth retardation, and sterility were induced with the expression of target genes [91]. Recently, this system was used to express the sodium transporter AtHKT1;1 in rice root cortical cells of a tissue marked line of the OTL library. The cortex-specific GFP-patterned rice plants transactivating the UAS AtHKT1;1 construct showed increased fresh weight under salinity stress, which was

related to a low concentration of Na(+) in the shoots. This work represented an important step in the development of abiotic stress tolerance in crop plants via targeted changes in mineral transport [124]. Other potential applications of the cell-specific transactivation include cell ablation of tissues through transactivation of a lethal gene, such as the diphtheria toxin gene, or RNAi-mediated gene silencing in specific cell types.

7 Conclusions and Prospects

Following major national initiatives that are now complete, a large mutant resource in rice has been generated by chemical, physical, or insertion mutagenesis in the last 15 years. Although the current size of the international collection of insertion lines (675,000) appears sufficient, the level of molecular characterization of this global resource, with only 226,000 sequence-indexed inserts anchored on the rice genome, still lags behind that of *Arabidopsis* (385,000). So far ~28,000 non-TE genes inserted over 38,000 predicted gene sequences (i.e., 72 %) harbor at least one sequence-indexed insert. In total, 30 % of the rice genes have at least three allelic insertions and therefore good probability to encompass a knockout line. The scientific community should intensify the FST generation effort. However, the organization of the T-DNA inserts in some lines and redundancy of *Tos17* may hamper fast progress in this area. Also, effort should be made to verify the quality of the FST indexation because both FST generation and seed increase are error-prone.

In the last few years, other graminaceous systems have been proposed to complement or replace the rice model, notably in countries with limited expertise in rice. These emerging sequenced models include *Brachypodium distachyon* and *Setaria italica*, which are short-cycle plant species that can be cultivated with limited greenhouse care and their seeds exchanged with little restrictive quarantine and Intellectual Property constraints. In rice, six different commercial cultivars have been chosen to generate insertion libraries (Nipponbare, Dong Jin, Hwa Young, Zhonghua 11 and 15 and Tainung 67) on the basis of the availability of their genome sequence, economic significance and/or tissue-culture amenability. This choice was not driven by an anticipation of their adaptation to greenhouse growth conditions or by the future need for sharing a unique genetic background. Recently, the use of Kitaake, a japonica cultivar grown in Hokkaido island, North Japan, with both a short cycle (8 weeks), photoperiod insensitiveness, a compact habit and transformation amenability, was proposed as a model genotype for rice functional genomics. Generating a new large sequence-indexed insertion Kitaake mutant resource in an effort shared by several laboratories has been proposed.

Next-generation sequencing of the insertion mutant resource might be an attractive complement to the FST generation effort. Recently, M4 lines derived from three Nipponbare regenerants of the *Tos17* NIAS collection have been sequenced at a coverage equivalent to 5.8, 6.2, and 16.2 times the size of the rice genome [100]. The frequency of homozygous SNPs, taking into account that 43.75 % of the genome returned to homozygosity in M4, were 1 SNP/153 kb (2,492), 1 SNP/368 kb (1,039) and 1 SNP/849 kb (450) depending on the line. These frequencies fell within the range of lesions observed in MNU-derived populations (1/350 kb [133] to 1/500 kb [140]). Therefore, a mutation rate of 1.74×10^{-6} base substitutions per site occurs during tissue culture. In *Arabidopsis*, the mutation rate of regenerated plants ranges from 60- to 350-fold that of the spontaneous mutation rate, estimated at 10^{-7}–10^{-8} events per base pair [52, 57]. The mutation spectrum of somaclonal variation was found similar to the spectrum of spontaneous mutations, with the exception of the relatively low level of C to T transitions [100]. Thus, DNA of T-DNA or *Tos17* insertion lines can be pooled to help detect lesions by TILLING on gel or by sequencing.

Unraveling by 2020 the function of the thousands of genes underlying the most important agronomic traits among the 38 K non-transposable element rice genes remains an ambitious goal for a concerted effort of the rice scientific community [159]. Use of fully characterized mutant resources is certainly pivotal and strategic to help

in reaching that objective and should not be constrained by any exchange restriction. A recent review estimated that the functions of 600 rice genes have been deciphered [52, 57]. Along with the effort of gene function elucidation by forward and reverse genetics approaches, the ongoing sequencing and evaluation of thousands of rice genotypes will quicken the association of nucleotide variation with phenotype and performance and will necessitate organizing rice functional analysis research in true pipelines of validation with collaboration at an international level.

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Transcriptome Profiling

8

Lei Li and Xing-Wang Deng

1 Introduction

Transcriptome refers to the full complement of RNA molecules produced in one or a population of cells in a specific developmental stage or physiological condition. Given the pivotal roles of RNA, elucidation of the transcriptomes associated with biological processes and developmental programs has been for decades a central theme in biology. Technological advances in recent years have led to the development or improvement of methods for analyzing transcriptome composition and dynamics with ever-increasing throughput, coverage, accuracy, and cellular resolution. The unprecedented scale and complexity of the transcriptomic data have transformed both experimental and computational biology. For the purpose of this chapter, we limit the discussion of rice transcriptomics to mRNA, as noncoding and small RNAs are the topics of other chapters.

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2 Transcriptome Composition

2.1 EST and FL-cDNA Sequencing

In many organisms, the first transcriptome approach is usually the collection and sequencing of a large number of expressed sequence tags (ESTs), which are useful for gene discovery and genome analysis. More than 1.2 million ESTs from rice have been registered in the NCBI dbEST database (http://www.ncbi.nlm.nih.gov/dbEST/). Owing to the fragmented nature of EST reads, however, they often do not contain the whole open reading frame (ORF) of an expressed gene. Therefore, full-length complementary DNA (FL-cDNA) collections are regarded as critical resources for post-genomic research, and techniques have been established to prepare FL-cDNA enriched libraries from various organisms. A major advantage of this approach over EST is that the complete ORFs as well as the 5′ and 3′ untranslated regions are obtained, which permits identification of gene structure. Availability of large-scale FL-cDNA sequences thus aids in accurate annotation of the sequenced genome [23, 62], helps in determining transcriptome composition [23, 57], and facilitates functional assays through homology search for conserved protein domains or motifs [62].

FL-cDNA clones from the *japonica* rice were mainly collected and sequenced through the Rice Full-Length cDNA Consortium of Japan, which was launched in 2000. The libraries were

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constructed from more than 50 different tissue types with and without stress treatments, using either the oligo-capping method [35] or the biotinylated cap trapper method [6]. In this project, more than 380,000 clones were collected and randomly sequenced from the 5′ and 3′ ends. The first report of this effort was published in 2003. This landmark report describes the characterization and annotation of 28,000 FL-cDNA sequences [23]. The collection has eventually expanded to 578,000 FL-cDNA clones and FL-ESTs, among which 35,000 cDNA clones were completely sequenced and annotated ([43]; http://cdna01.dna.affrc.go.jp/cDNA/).

For the *indica* subspecies, over 20,000 putative FL-cDNAs were isolated from two varieties, Guangluai 4 and Minghui 63 ([29, 31]; http://www.ncgr.ac.cn/ricd), as part of the National Rice Functional Genomics Project of China. Sequence analysis of the 10,096 FL-cDNAs derived from Guangluai 4 revealed that 9,029 match annotated gene models while 1,200 are possibly new transcription units [29]. Interestingly, comparison with the *japonica* cDNA collection indicated that, of the cDNAs present in both *indica* and *japonica*, 3,645 (46 %) exhibit substantial differences at the protein level due to single-nucleotide polymorphisms (SNPs), small insertions/deletions, or sequence-segment variations [29]. As these variations might be associated with the phenotypic changes of the two cultivated rice subspecies, they highlight the importance of comparative approaches in the study of rice transcriptomics.

Rice is the first monocot plant with its genome fully sequenced and annotated. The high-quality genome sequences and annotations constitute an enormously useful tool for research in crop plants. However, genome annotation for protein- and nonprotein-coding genes is a reiterative process that heavily depends on experimental support such as FL-cDNA collections. Annotation of the rice genome has evolved significantly since release of the draft sequences as resources accumulate [40, 62]. In a comprehensive analysis, Satoh et al. [43] cross-referenced 28,500 FL-cDNA-supported loci in the rice genome with the predicted genes [40]. They classified rice genes into 23,000 annotated expressed (AE) genes, 33,000 annotated non-expressed (ANE) genes, and 5,500 non-annotated expressed (NAE) genes, based on the annotation and the presence of corresponding cDNA sequences. Further, NAE and ANE genes differ considerably from AE genes regarding the correlation to expression status determined from microarray analysis and gene structures. Collectively, their results indicate that rice genes could be divided into distinct groups based on transcription level and gene structure and that coverage bias of FL-cDNA clones exists due to transcription activity and other factors [43].

2.2 Genomic Tiling Microarray Analysis

In addition to EST and FL-cDNA sequencing, genomic tiling microarrays were used to systematically identify components of the rice transcriptome. Tiling microarrays involve the generation of a virtual tile path made up of progressive oligonucleotide probes that collectively represent a target genome region or the entire genome [38]. The average number of nucleotides between the centers of neighboring probes, called the step, defines the resolution of a tiling microarray. Advances in high-density oligonucleotide microarray technologies, which now allow several hundred thousand to several million distinct oligonucleotide probes to be synthesized on a single array, make it feasible to tile even complex genomes at high resolutions. The entire probe sets are used to hybridize to fluorescence-labeled RNA samples. Hybridization intensity of each probe can be retrieved and analyzed to identify transcribed regions of the genome, independent of a priori annotations.

In rice, tiling microarray analysis started with chromosomes 4 and 10 [17, 27], which were among the first batch of chromosomes to be completely sequenced. The chromosome 4 tiling microarray was made of PCR-generated overlapping probes that were 3 kb long on average. By examining six organ types representative of rice developmental stages, the researchers detected expression of over 80 % annotated genes

of the chromosome and found 1,643 additional transcribed loci [17]. Comparison of chromosome-wide transcription activity with cytologically defined chromatin states revealed that euchromatin was more actively transcribed than the heterochromatin in the juvenile stage. Conversely, increased transcription of transposon-related genes was observed in the reproductive stage [17].

Rice chromosome 10 [27] and subsequently both the *indica* [26] and *japonica* genomes [25] were analyzed by oligonucleotide tiling microarrays developed on the NimbleGen platform. These microarrays contained 36 oligonucleotide probes separated on average by 10 nucleotides and were hybridized with a pooled mRNA population derived from seedling root, seedling shoot, panicle, and suspension-cultured cells. The hybridization signals were systematically analyzed, which provided transcription support to the majority of annotated gene models. For example, of the 43,914 non-transposable element protein-coding gene models predicted from the improved *indica* whole genome shotgun sequences [62], transcription of 35,970 (82 %) was detected in the tiling microarray analysis [26].

Consistent with results in other model organisms, tiling microarray analysis detected significant transcription activities in the annotated intergenic regions of the rice genome. Systematic scoring of the tiling microarray data using stringent criteria identified many novel transcriptionally active regions (TARs) in the intergenic regions that were validated by several independent experimental means [26, 27]. A total of 25,352 and 27,744 TARs not encoded by annotated exons were identified in *japonica* and *indica*, respectively [25]. These TARs were found to account for approximately two thirds of the transcription activity detected by tiling microarrays and represent transcripts likely conserved between the two subspecies [25]. Subsequently, a portion of these TARs were identified as derived from duplicated gene fragments in the genome [53]. Together, results from these analyses indicate that the novel TARs compositionally resemble the exonic regions and thus provide reliable estimations of additional transcriptomic components beyond the predicted coding and noncoding genes. To catalogue all species of transcripts and to determine the transcriptional structure of these transcripts will remain as the key aims of rice transcriptomics.

3 Transcriptome Dynamics

3.1 Microarray

Various experimental procedures to map and quantify the transcriptome are principally based on either hybridization or sequencing approaches. First described by Schena et al. [44], microarray became the method of choice for high-throughput analysis of global gene expression in the late 1990s [1]. Such analyses typically involve hybridization of the appropriate arrays to fluorescently labeled targets, which can be either cDNA or cRNA, with or without intervening amplification steps. The earlier microarrays contained cDNA elements such as PCR amplicons or EST clones representing known or predicted genes. Now the elements are synthesized as single-stranded oligonucleotides 30–70 bases in length. In rice transcriptome studies conducted so far, several different methods were employed to construct the arrays.

One of the earliest rice expression arrays was developed by the Rice Microarray Project of Japan, which was a 1,265-element PCR amplicon array prepared from EST clones [59]. This array was later updated to 8,987 elements [60]. Several other spotted cDNA arrays were developed around the same time. For example, a 1,728-element array was used to examine gene expression during the initial response to high salinity [21]. Later, a 10 K-element array was constructed and extensively used for profiling transcriptome changes associated with pollination and fertilization [24], during anther development [55], in the early stage of nitrogen starvation [28], and in hybrid rice [16]. Based on FL-cDNAs [23], Agilent developed a 22,000-elment array containing 60-mer probes, which was first used to detect abscisic acid- and gibberellin-induced genes in rice [61]. Using the same array, Furutani et al. [8] examined differentially expressed genes during panicle development.

These early transcriptome-profiling efforts were helpful to verify annotated gene models, to facilitate functional analysis of predicted genes, and to generate candidate transcripts associated with specific processes. However, the arrays contained probes that only profile portions of the transcriptome. Development of true whole genome microarrays was highly desired to more comprehensively and effectively study the transcriptome. A whole genome microarray would allow the generation of a reference expression dataset by different researchers and serve as a starting point for cataloguing transcriptome activity throughout the life cycle of rice, under treatments soliciting specific biological responses, or in mutants disrupting various pathways. Such gene expression compendia would aid in the identification of expression programs and elucidation of the underlying regulatory networks.

For *japonica* rice, an oligonucleotide near-whole genome array was constructed based on annotated gene models that have EST and/or FL-cDNA support. This array contains 43,312 oligonucleotide probes corresponding to 44,974 *japonica* rice transcripts [20]. This array was first used to compare expression profiles of light- and dark-grown rice leaf tissue [20]. In profiling gene expression during cold acclimation, Yun et al. [63] observed transcriptomic changes typical of oxidative response based on similarities to disease, elicitor, or wounding induced processes. It should be noted that similarities of gene expression profiles often provide important clues to the biological functions of genes and pathways. There are resources such as the database OryzaExpress [11] developed to facilitate the integration of information from genome annotations, transcriptome, and metabolic pathways for rice research.

For *indica* rice, a 70-mer microarray covering 41,754 annotated *indica* genes was first developed to analyze the transcriptome in six representative organs [34]. A large percentage of the predicted genes lacking significant homology to *Arabidopsis* genes were found to be expressed [34]. Using the same array, gene expression profiles regulated by light signaling [18] and during the development of somatic embryos and other organs [49] were investigated. As one of the early comparative analyses based on microarray data, Jiao et al. [18] performed global comparison of expression profiles between rice and *Arabidopsis*. This analysis revealed a higher correlation of transcript levels of gene expressed in constant light than in darkness, suggesting that the transcriptome related to photomorphogenesis is more conserved [18].

Rice oligonucleotide arrays were also developed on the Affymetrix GeneChip platform, which set the standard of microarray analysis in *Arabidopsis*. Early works using the rice GeneChip array included studies to profile transcriptome activities associated with grain filling [65] and drought tolerance [12]. Bethke et al. [3] examined gene expression patterns in aleurone cells subjected to different treatments. They noted that aleurone of a dwarf1 mutant exhibited dampened transcriptomic changes compared to wild-type aleurone undergone the same treatments [3]. Most recently, the whole genome GeneChip array was used to characterize the transcriptional programs controlling rice seed development. Xue et al. [56] analyzed the transcriptome of developing embryo, endosperm, and seeds under normal and chilling temperatures. They found that genes associated with transcriptional regulation, signaling pathways, and metabolic pathways were involved in the response to low temperature in early stages of seed development. In a landmark study for expression levels of quantitative trait loci, Wang et al. [52] used GeneChip arrays to profile germinating shoots from 110 recombinant inbred lines, providing a new perspective on elucidating the regulatory networks.

In the vast majority of microarray experiments discussed above, RNA was typically prepared from biological samples that contain many mixed cell types and consequently provide limited spatial resolution [9]. Increasing the spatial resolution requires separation of organs or tissues into their constituent cell types prior to RNA isolation. Two major techniques, namely, fluorescence-activated cell sorting [4] and laser capture microdissection [2], were developed to isolate specific cell types for transcriptome profiling in plants. Using laser microdissection and microarray profiling, Jiao et al. [19] produced a cell-specific

transcriptome atlas in rice. This atlas included 40 distinct cell types from shoot, root, and germinating seed at several developmental stages. Profiling and comparison of the steady state transcript levels in a cell-specific fashion uncovered coexpressed genes and pathways, cell-specific promoter motifs, candidate interaction partners, and putative hormone response centers [19]. As more cells and conditions are added, it should be possible to use this approach to distinguish an invariant expression fingerprint for each cell type that yields insights into the regulatory networks underpinning cell differentiation.

3.2 RNA Sequencing

In contrast to microarray methods, sequence-based approaches measure the copy number of transcripts by directly determining the cDNA sequences. In the past, reliance on Sanger sequencing made most of these approaches preventively expensive and cumbersome in annotating the structure of transcriptomes. Recently, development of next-generation DNA sequencing technologies has provided a new method for measuring both the presence and prevalence of transcripts. This method, termed RNA sequencing (RNA-Seq), is modeled after the success of whole genome shotgun sequencing. In general, a population of RNA (total or fractionated) is reverse transcribed to cDNA fragments. With adaptors attached to one or both ends, the cDNA fragments are converted to a library with or without amplification. The library is then deeply sampled in a high-throughput manner using one of several next-generation sequencing platforms [54]. Although the obtained reads are generally short (tens to hundreds of nucleotides, depending on the DNA sequencing technology used), the massive parallel nature of the reads ensures that the transcriptome is well represented and sufficiently covered.

RNA-Seq has clear advantages over existing transcriptome-profiling approaches. Studies based on this method have already enhanced our view of the complexity and dynamics of eukaryotic transcriptomes [39, 46, 54]. Deep coverage provided by RNA-Seq offers base-level resolution that can reveal the precise location of transcription boundaries. This feature is valuable to determine the transcription start sites, polyadenylation sites, as well as alternative splicing events and makes RNA-Seq desirable for studying complex transcriptomes. In one of the first studies in rice applying RNA-Seq, Lu et al. [32] validated 83 % of the current rice gene models and identified 6,228 gene models that can be extended at the 5′ and/or 3′ ends by at least 50 base pairs. Further, they found that almost half of rice genes are alternatively spliced, considerably more than previous estimations [32]. This finding was corroborated by a similar study [64], which also documented more than 200 putative chimeric transcripts produced by trans-splicing. In analyzing the transcriptomes in a wide range of tissues at various developmental stages or exposed to environmental treatments, a striking observation was made that 80 % of rice genes may use alternative polyadenylation sites [45].

Unlike microarray analysis, which is limited to monitoring the expression of previously annotated genes that have corresponding probes on the array, RNA-Seq can detect novel transcripts. For example, global sampling of the transcripts in both the *indica* and *japonica* subspecies produced 15,708 novel TARs from the piling up of short reads mapped on the genome, more than half of which have no homolog to known proteins [32]. Interestingly, more than 60 % the novel transcripts identified by tilling array analysis [25] were detected by an RNA-Seq experiment [64]. In an RNA-Seq analysis to monitor the whole transcriptome of salinity stress-treated rice tissues, Mizuno et al. [37] identified 2,795 (shoot) and 3,082 (root) currently unannotated transcripts. It was reported that some of these unannotated transcripts likely encode functional proteins and that they are differentially expressed in response to salinity stress [37].

Regarding transcriptome dynamics, RNA-Seq provides a more precise measurement of transcript levels than other methods. Unlike microarray analysis, RNA-Seq does not suffer from an upper limit for quantification of transcript abundance, which correlates with the number of reads

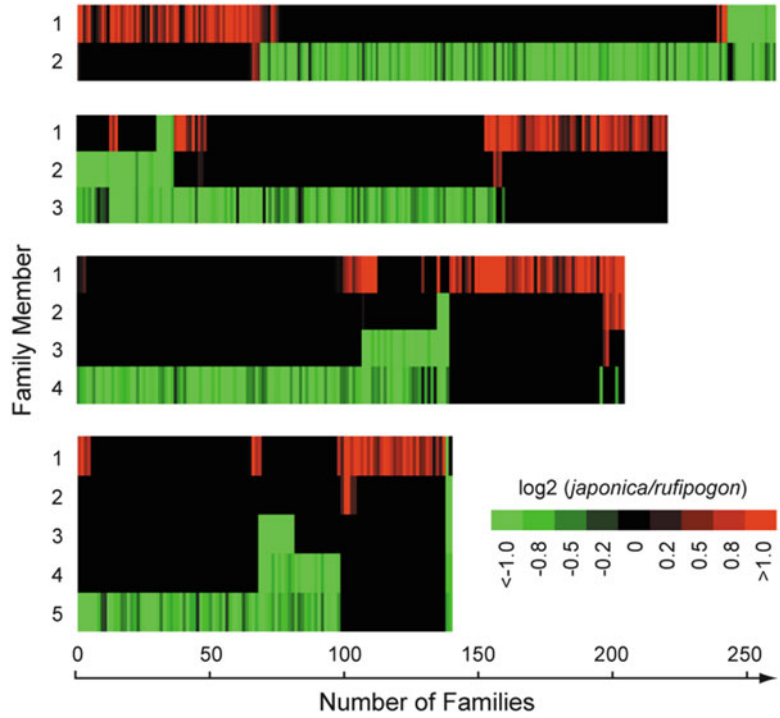

Fig. 8.1 Differentially expressed genes in paralogous gene families. Gene expression was determined for rice lineages *japonica* and *rufipogon*. Relative expression level of differentially expressed genes belonging to paralogous families (only families with 2–5 members are shown) was calculated and color coded. Each family member is shown as a bar with each column representing a paralogous family. Modified from Peng et al. [41]

that can be adjusted according to the experimental needs. Consequently, it offers a much larger dynamic range over which to measure gene expression levels. In studies performed in yeast and mammals, RNA-Seq has been shown highly accurate for quantifying transcript levels, as determined by quantitative PCR analysis and spike-in RNA controls [50, 54]. Compared to microarrays, the results of RNA-Seq were found to be highly reproducible for both technical and biological replicates and even across laboratories [50, 54]. In the handful of RNA-Seq studies performed in rice, Zhang et al. [64] already reported unambiguous detection of transcripts expressing at an extremely low level using paired-end RNA-Seq. Taking all of the technical advantages into account, RNA-Seq allows the entire transcriptome to be surveyed in a quantitative manner and at a relative low cost, providing researchers with efficient ways to measure transcriptome dynamics.

Another area RNA-Seq would be increasingly useful is comparative analysis of transcriptomic data from different species. Just as comparative genomics has been instrumental in genome annotations, comparative analysis of transcriptomes should prove valuable in elucidating gene regulation and evolution. Identification of orthologous genes among different species and large-scale transcriptomic data are necessary for the comparative efforts. To compare transcriptome in the heading-stage panicle, Peng et al. [41] performed microarray analysis in six lineages of cultivated and wild rice. The researchers detected a large portion of the transcriptome that exhibited differential expression between Asian cultivated and wild rice. The authors showed that downregulation of gene expression was the dominant trend in cultivated rice, which was achieved largely by repression of members of paralogous gene families (Fig. 8.1).

While the study by Peng et al. [41] benefited from the close relatedness of the rice lineages, inclusion of other species would have made the design of probes problematic. In this regard, RNA-Seq is more readily applicable as it independently measures the transcriptomes of different species. More recently, Davidson et al. [7] employed RNA-Seq to compare analogous transcriptomes in the reproductive tissues of three representative grass species including *Brachypodium distachyon* (Pooideae), sorghum (Panicoideae), and rice (Ehrhartoideae). This analysis revealed that only a fraction of orthologous genes exhibit conserved expression patterns. They show that genes with narrow expression domains have accelerated rates of sequence evolution than the highly and broadly expressed genes. Further, orthologous genes in syntenic genomic blocks tend to have correlated expression compared to non-syntenic orthologs [7].

4 Allele-Specific Gene Expression

When more than one set of alleles are present in the same organism, it is assumed in many transcriptome-profiling efforts that the alleles contribute equally to the transcript pool and hence only the sum should be measured. In diploid eukaryotes, it is clear that many genes are not equally expressed from the paternal and maternal alleles [5, 36, 47]. At the extreme are the imprinted genes exclusively transcribed from the non-silenced allele. In a recent effort to identify imprinted genes in rice seed, Luo et al. [33] analyzed RNA from embryo and endosperm of seeds obtained from reciprocal crosses between the *japonica* and *indica* subspecies. Sequenced reads from cDNA libraries baring SNPs were counted to estimate parental expression bias. Statistical analysis revealed 262 candidate imprinted loci in the endosperm and three in the embryo, which were not clustered in the genome as found in mammals [33]. Interestingly, some of the imprinted genes are also expressed in vegetative tissues of rice, suggesting that they may have additional function in plant growth.

Studies in human revealed that up to 50 % genes may exhibit imbalanced allele expression in somatic cells [5, 22, 36, 58]. Investigations in plants also indicated that allele-specific expression is prevalent in F_1 hybrids [10, 47, 48]. As variation in other regulatory genes and environmental influence are both controlled for the set of alleles present in the same cell, allele-specific expression can largely be attributed to variation in the regulatory regions [22]. These studies thus suggest that allele-specific expression provides a useful readout for linking coding polymorphisms to regulatory polymorphisms.

Much of the effort to experimentally distinguish the transcript of one allele from its highly similar counterpart has been directed toward SNP, the most abundant form of DNA polymorphism. Experimental procedures based on different principles to examine unequal transcription from SNP-defined alleles include microarray-based applications. Allele-specific microarrays [30, 42, 51] were developed as a high-throughput, multiplex, target-oriented platform for globally quantifying unequal allelic expression from genes of interest. These microarrays typically involve pairs of probes with perfect match to one of the two alleles, based on the idea that a sequence mismatch may significantly disrupt hybridization and attenuate a probe's signal to the nonmatching target. However, sensitivity, specificity, and linearity of the allele-specific probes' response to allele-specific transcript levels all need to be experimentally tested.

To further develop allele-specific microarrays, He et al. [15] selected 518 SNPs between two rice subspecies *indica* and *japonica* and investigated the potential of tiling microarray in detecting and quantifying allele-specific expression. Schematic of the tiling strategy is illustrated in Fig. 8.2, which involves 25 blocks of 25-mer probes for each SNP. Within each block, there are two probes that match perfectly to the *indica* and *japonica* alleles and two probes that each contain one of the two remaining nucleotides. A titration series consisting of five cDNA mixtures in which the *japonica*- and *indica*-derived cDNA templates were mixed proportionally and hybridized to the SNP-based tiling arrays. For each block, the ratios of

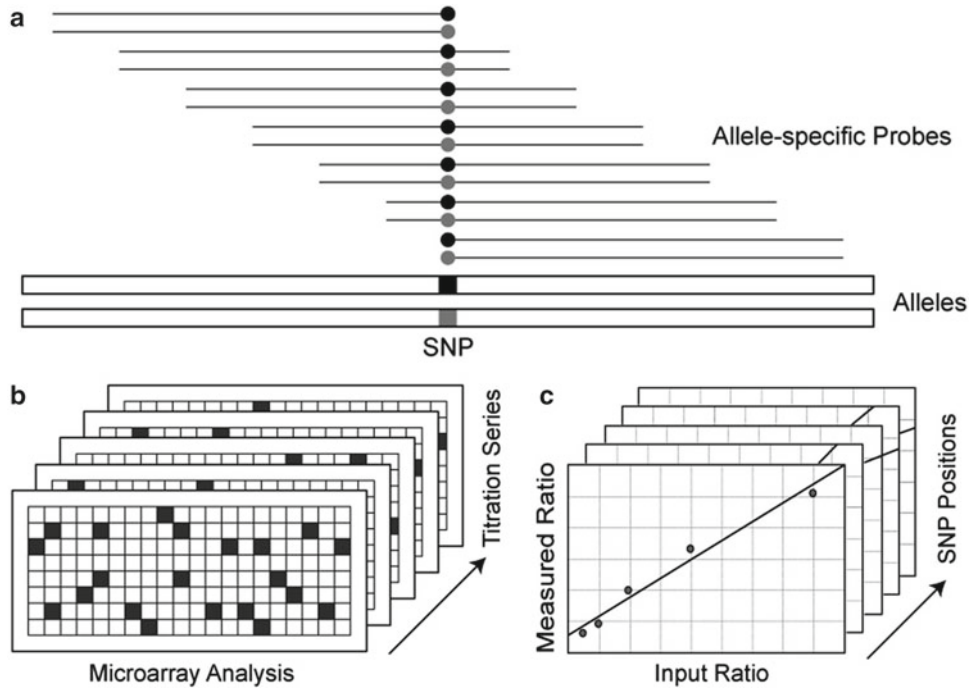

Fig. 8.2 Schematic of the tiling microarray approach to screen for probe blocks that distinguish allele-specific transcripts. (**a**) For each SNP represented on the tiling microarray, 25 blocks of 25-mer oligonucleotide probes are designed. Within each block, four probes representing all four possible nucleotides at the SNP site are included. Two of these probes match perfectly to the *indica* and the *japonica* allele (allele-specific probes), while the other two are included as mismatch controls (not shown). (**b**) Identical tiling microarrays are hybridized to a series of targets in which the *japonica*- and *indica*-derived cDNA templates are mixed in various predetermined proportions. (**c**) The probe blocks representing each SNP are screened based on a regression model for those that show significant linearity between the measured and the input ratios of allele-specific transcripts. Modified from He et al. [15]

indica- and *japonica*-specific transcript were compared to the input ratios using a regression model. Based on a cutoff for significant linearity, over half of the SNPs were found to be able to accurately quantify allele-specific transcripts [15]. By testing the leaves of the reciprocal *japonica*×*indica* hybrids, approximately one third of the SNPs were found to be unequally represented in the transcript pool and some were linked to sequence variations in the promoters [15].

RNA-Seq is also a powerful method for detecting allele-specific expression. In a comprehensive analysis of the transcriptomes of *japonica* and *indica* rice as well as their reciprocal hybrids, He et al. [14] used RNA-Seq to assess differential allelic expression in relationship to epigenetic modifications. Using stringent criteria, 2,205 SNPs contained in 1,754 genes were selected for evaluating allele-specific biases by a binomial test. It was found that 398 SNPs (328 genes) show either an allelic bias for accumulated transcripts or epigenetic modifications in both reciprocal hybrids. A high correlation of allelic bias of epigenetic modifications or gene expression in reciprocal hybrids with their differences in the parental lines was observed. Further, comparison of the ratio of two parental alleles revealed that almost all affected genes exhibit the same direction of allele-specific bias in expression or epigenetic modifications in both reciprocal hybrids, suggesting that there is no significant parent-of-origin effect [14].

5 Conclusion and Perspective

Transcriptome profiling has become indispensable in biology. Studies performed in rice have facilitated genome annotation, elucidated portions of the regulatory networks, and produced many target genes differentially regulated in specific processes. As new technologies promise to improve the scope and quality of transcriptomic data, we will continue to face the same challenges. To fully decipher the rice transcriptome, systems-based approaches are highly desirable to integrate the expression profiles with data characterizing other functional elements of the cell such as global changes of histone modifications [13]. The integrated data, in conjugation with modeling and model testing, should provide important clues to the regulatory networks that program genome transcription, function, and adaptation in rice. Elucidation of the regulatory networks should in turn generate biologically meaningful interpretations and predictions to drive follow-up experimentation that accelerates functional discovery in this important crop.

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Epigenomics

9

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In eukaryotes, the genomic DNA is tightly compacted into chromatin, the structure of which plays essential roles in genome function and gene expression [1]. The primary unit of chromatin is the nucleosome. The nucleosome core particle is comprised of histone H2A, H2B, H3, and H4, and is wrapped around by a segment of 147 bp DNA. Chromatin modifications include DNA methylation and histone modifications. DNA methylation in eukaryotes consists of the addition of a methyl group at position five of the pyrimidine ring of cytosine [2]. Histone modifications include acetylation, methylation, phosphorylation, and monoubiquitination, etc. DNA methylation and histone modifications are reversible and are recognized and bound by different chromatin protein complexes that usually have chromatin remodeling activities to alter chromatin structure [3, 4]. Chromatin modification profiles define distinct epigenomes which are reflected by specific gene expression patterns of different cell types and/or responses to variable environmental conditions. Epigenetic regulations involving variation of DNA methylation and histone modifications and histone variant deposition, etc., control transcriptional activity of genes, repetitive sequences and transposable elements, as well as DNA replication and repair [5]. In this chapter, we will describe recent advances in studies of rice chromatin modification, regulation and recognition mechanisms, and their function in controlling rice gene expression and plant growth.

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1 DNA Methylation in Rice

DNA methylation is a hall mark of epigenetic inactivation of repetitive sequences and transposable elements and heterochromatin formation in plants. In plant genomes, cytosine methylation occurs in CG, CHG, and CHH contexts (where H is A, C, or T). DNA methylation is highest within pericentromeric regions that are enriched for transposable elements and repetitive sequences including ribosomal DNA (rDNA). However a significant proportion of genes (15–20 %) also contain methylated cytosines in *Arabidopsis* and rice [6–9]. Methylation of CG sequences is commonly found within gene bodies, whereas methylation of non-CG (CHG and CHH) sequences is enriched in transposons and repetitive sequences. In genes, DNA methylation is distributed over the transcribed regions or gene bodies but in most cases is depleted from the 5′ and 3′ ends of the genes (Fig. 9.1). It is suggested that moderately

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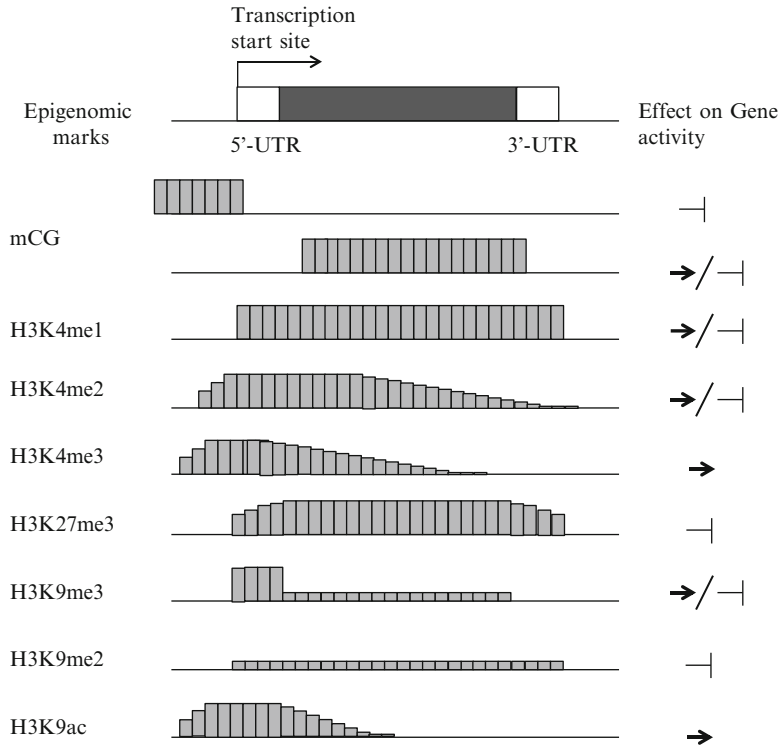

Fig. 9.1 Correlation of epigenomic modifications and gene activity. Distribution of indicated marks along the gene is shown. *Arrows*: transcriptional activation, *bars*: repression

expressed genes are more likely to be methylated than those with low or high expression [10]. Gene body methylation (i.e., CG methylation) is conserved between plants and animals and is hypothesized to suppress spurious initiation of transcription within active genes [7, 11]. About 5 % genes show DNA methylation within promoter regions, which has a repressive function on promoter activity. DNA methylation can inhibit transcription or lead to silent chromatin either by physically impeding the binding of transcription factors to the promoter or by interacting with methylcytosine-binding proteins that can recruit additional chromatin proteins to the locus to modify histones or remodel the chromatin thereby forming compact heterochromatin.

In *Arabidopsis*, CG methylation is maintained by the DNMT1 (DNA MethylTransferase 1) homologue, MET1 (Methyltransferase1), CHG methylation primarily by the plant-specific DNA methyltransferase CMT3 (ChroMomeThylase 3), and CHH methylation by DMR2 (Domains Rearranged Methyltransferase 2), a homologue of mammalian DNMT3 [12] (Fig. 9.2). In addition, DRM2 is responsible for de novo methylation of all three sequence contexts, which is directed by small interfering RNAs (siRNAs) called RNA-dependent DNA methylation (RdDM) [13]. While a general concept is that distinct DNA methyltransferases are responsible for either maintenance or de novo methylation in different sequence contexts, an emerging view is that different enzymes may cooperate to catalyze both steps. Rice genes encoding putative DNA methyltransferases and the siRNA machinery have been identified (Table 9.1). Loss-of-function mutations of rice DNA methyltransferase genes lead to reduction of DNA methylation of repetitive sequences (unpublished). DNA methylation can be lost by passive (non-maintenance during DNA replications) and active (enzymatic removal) mechanisms. *Arabidopsis* DNA demethylase DME (Demeter) and ROS1 (Repressor Of Silencing 1) have combined DNA

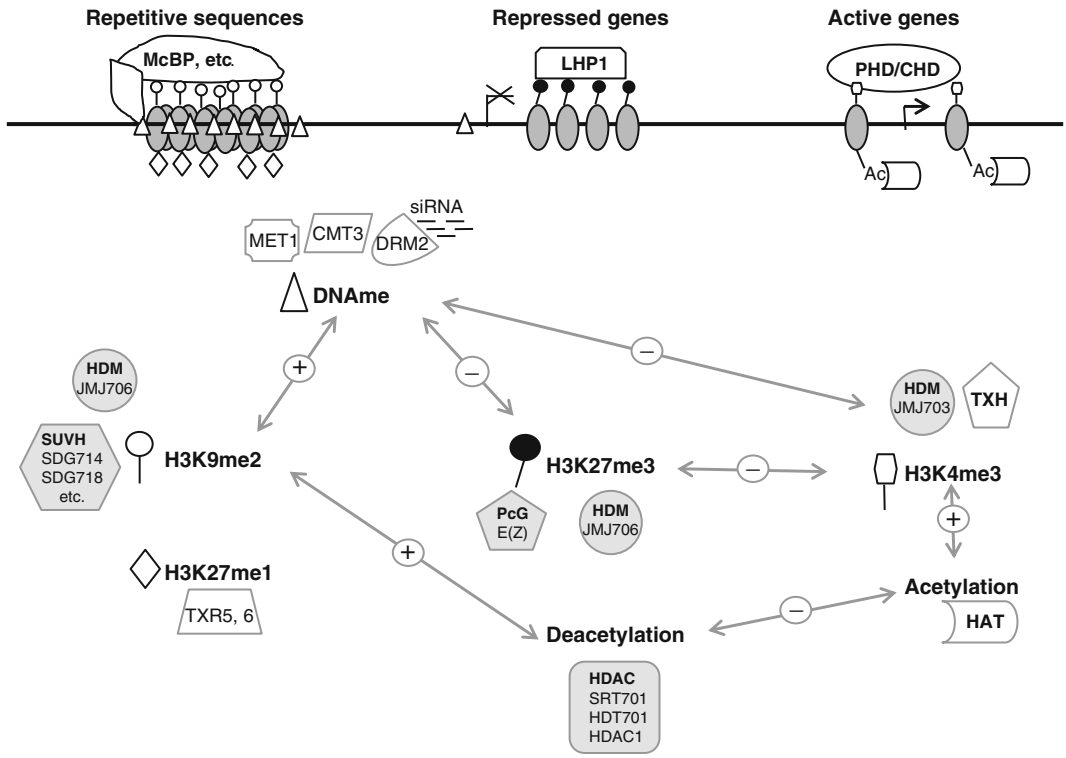

Fig. 9.2 Schematic representation of chromatin structures over repetitive sequence, repressed, and active genic regions. In repetitive sequence regions, cytosines are methylated at CG, CHG, and CHH sequence contexts. H3K9me2 and H3K27me1 are enriched. These modification marks are bound by proteins associated with heterochromatin such as McBP (methylated Cystosine Binding Proteins, etc.) and nucleosomes are highly condensed. In repressed genic regions histones are deacetylated and H3K27me3 is enriched which is bound by LHP1. In active genic regions, histone acetylation level is high and H3K4me3 is enriched at 5′ end of genes. Acetylated histone lysines are recognized by bromodomain-containing proteins (such as HAT) that have transcription coactivator function. H3K4me3 can be recognized by PHD or CHD proteins that facilitate transcription by RNA polymerase II. In plants, DNA methylation (DNAme) is catalyzed by three types of enzymes: MET1, CMT3 and DRM2. De novo DNA methylation mediated by DRM2 is trigged by siRNA. DNAme and H3K9me2 that is regulated by the SUVH (Su(var) homologue) class of histone methyltransferases (HMT, i.e., SDG714/718 in rice) and histone demethylase (HDM, i.e., JMJ706 in rice) are mutually agonistic. H3K27me3 is mediated by the E(Z) type of HMT which is the key component of the polycomb group (PcG) complexes and demethylated by HDM (probably JMJ706 in rice). H3K27me3 is antagonistic to H3K4me3 and DNAme. H3K4me3 is catalyzed by the trithorax homologue (TXH) proteins and demethylated by JMJ703 in rice. H3K4me3 inhibits DNAme and H3K27me3. Histone (mostly H3 and H4) lysines are acetylated by HAT (histone acetyltransferases) and deacetylated by HDAC (histone deacetylases, such as SRT701, HDAC1, and HDT701 in rice). Histone acetylation facilitates H3K4me3, while histone deacetylation facilitates H3K9me2. H3K27me1 is catalyzed primarily by TXH-related (TXR5, 6) proteins

glycosylase and AP lyase activities [14, 15]. The rice DNG701 protein that is closely related to *Arabidopsis* ROS1 has been shown to display 5-methylcytosine DNA glycosylase and lyase activities in vitro [16]. Knockout or knockdown of *DNG701* in rice leads to DNA hypermethylation and reduced expression of the retrotransposon *Tos17* [16].

2 Histone Modifications in Rice

2.1 Histone Acetylation/ Deacetylation

Strong acetylation of histones induces relaxation of chromatin structure and is associated with transcriptional activation, whereas weak or no

Table 9.1 Rice chromatin modification and remodeling genes

	Class	ChromDB name	Tigr locus		Class	ChromDB name	Tigr locus
DNA methyltransferase	MET1	DMT702	Os03g58400	SiRNA-related	Dicer-like	DCL701 [84]	Os03g02970
		DMT707	Os07g08500			DCL702	Os01g68120
	DRM1/2	DMT705	Os01g42630			DCL703 [85]	Os10g34430
		DMT706	Os03g02010			DCL704 [85, 86]	Os04g43050
		DMT708	Os12g01800			DCL705 [87]	Os03g38740
		DMT709	Os11g01810				Os09g14610
		DMT710	Os05g04330		Argonaute	AGO701	Os02g58490
	CMT3	DMT701	Os03g12570			AGO702	Os06g39640
		DMT703	Os05g13790			AGO703	Os01g16870
		DMT704	Os10g01570			AGO704	Os07g28850
DNA demethylase		DNG701	Os05g37350			AGO705	Os04g06770
		DNG702	Os01g11900			AGO706	Os03g58600
		DNG703	Os02g29230			AGO707	Os06g51310
		DNG704	Os05g37410			AGO708	Os04g47870
Methyl binding domain protein		MBD701	Os09g32090			AGO709	Os04g52540
		MBD703	Os06g48870			AGO710	Os03g47830
		MBD704	Os04g20560			AGO711	Os02g45070
		MBD705	Os04g19690			AGO712	Os07g09020
		MBD706	Os05g33550			AGO713	Os04g52550
		MBD707	Os12g42550			AGO714	Os03g33650
		MBD708	Os09g29750			AGO715	Os07g16230
		MBD709	Os08g37920			AGO716	Os02g07310
		MBD710	Os02g09920			AGO717	Os03g47820
		MBD711	Os05g33550			AGO718	Os03g57560
		MBD712	Os04g11640		RNA-dependent RNA polymerase	RDR701	Os01g34350
		MBD713	Os04g11730			RDR702	Os01g10130
		MBD714	Os04g11510			RDR703	Os01g10140
		MBD715	Os08g37900			RDR704	Os02g50330
		MBD716	Os04g11530			RDR705	Os04g39160
		MBD717	Os04g52380		RNA polymerase IV (large)	NRPDA701	Os04g48370
		MBD718	Os08g10580			NRPDA702	Os09g38290

Histone variant	H2A.Z	HTA705	Os10g28230			NRPDA703	Os02g05880
		HTA712	Os03g06670		RNA polymerase IV (small)	NRPDB701	Os04g54840
		HTA713	Os03g53190			NRPDB702	Os08g07480
	H2A.X	HTA704	Os03g51200				
		HTA711	Os12g34510				
	H3.3	HTR701	Os06g06480				
		HTR709	Os02g25910				
		HTR710	Os02g25940				
		HTR711	Os03g27310				
		HTR712	Os06g04030				
		HTR714	Os12g22650				
		HTR715	Os12g22680				
	CENPA	HTR707	Os05g41080				
Histone methyltransferase (SET domain)	E(Z) [46]	SDG711	Os06g16390	Histone acetyltransferase	GNAT	HAG702	Os10g28040
		SDG718	Os03g19480			HAG703	Os04g40840
	ATXH + ATXR	SDG723	Os09g04890			HAG704	Os09g17850
		SDG721	Os01g11950		MYST	HAM701	Os07g43360
		SDG705	Os01g46700		CBP	HAC701	Os01g14370
		SDG739	Os03g07260			HAC703	Os02g04490
		SDG722	Os04g53700			HAC704	Os06g49130
		SDG701	Os08g08210		TAF_{II} 250	HAF701	Os06g43790
		SDG741	Os10g27060	Histone deacetylase [21]	RPD3/HDA1	HDA701	Os01g40400
		SDG720	Os01g73460			HDA702 [25]	Os06g38470
		SDG730	Os02g03030			HDA703 [25]	Os02g12350
		SDG717	Os12g41900			HDA704	Os07g06980

(continued)

Table 9.1 (continued)

	Class	ChromDB name	Tigr locus		Class	ChromDB name	Tigr locus
	ASHH+ASHR	SDG724	Os09g13740			HDA705	Os08g25570
		SDG708	Os04g34980			HDA706	Os06g37420
		SDG725	Os02g34850			HDA707	Os01g12310
		SDG716	Os03g49730			HDA709	Os11g09370
		SDG740	Os08g10470			HDA710 [25]	Os02g12380
		SDG736	Os02g39800			HDA711	Os04g33480
		SDG707	Os08g34370			HDA712	Os05g36920
	SUVH+SUVR [38]	SDG704	Os11g38900			HDA713	Os07g41090
		SDG713	Os03g20430			HDA714	Os12g08220
		SDG709	Os01g59620			HDA716	Os05g36930
		SDG728	Os05g41170		HD2	HDT701 [20]	Os05g51830
		SDG733	Os11g03700			HDT702	Os01g68104
		SDG734	Os12g03460		SIR2	SRT701 [28]	Os04g20270
		SDG726	Os07g25450			SRT702	Os12g07950
		SDG715	Os08g45130	Histone ubiquitination protein	Group A	HUPA701	Os04g46450
		SDG714 [42]	Os01g70220		Group B	HUPB701	Os05g08960
		SDG703	Os04g34990			HUPB702	Os03g57790
		SDG710	Os08g30910			HUPB703	Os07g07240
		SDG727	Os09g19830	Chromatin remodeling factor (SNF2 domain) [89]	Snf2	CHR707	Os02g02290
		SDG706	Os02g47900			CHR719	Os05g05230
		SDG729	Os01g56540			CHR720	Os06g14406
		SDG742	–		ISWI	CHR727	Os05g05780
		SDG712	Os02g40770			CHR728	Os01g27040
	Others	SDG738	Os04g34610		CHD	CHR702	Os06g08480
		SDG731	Os07g28840			CHR703	Os01g65850
		SDG732	Os09g38440			CHR705	Os07g46590
Histone arginin methyltransferase [88]	OsPRMT1	PRMT703	Os09g19560			CHR729 [50a]	Os07g31450
	OsPRMT3	PRMT710	Os07g44640		DDM1	CHR741	Os03g51230
	OsPRMT4	PRMT702	Os07g47500			CHR746	Os09g27060
	OsPRMT5	PRMT708	Os02g04660		Others	CHR701	Os02g06592

	OsPRMT6a		Os10g34740	CHR704	Os01g01312
	OsPRMT6b		Os04g58060	CHR706	Os01g57110
	OsPRMT7	PRMT709	Os06g01640	CHR708	Os01g72310
	OsPRMT10		Os06g05090	CHR709	Os02g46450
Histone demethylase (Jumomji family)	KDM5/IARID	JMJ703 [60a]	Os05g10770	CHR710	Os02g32570
		JMJ704	Os05g23670	CHR711	Os03g01200
		JMJ708	Os06g51490	CHR712	Os04g59620
	KDM4/JMJD2	JMJ706 [49]	Os10g42690	CHR713	Os05g15890
		JMJ707	Os02g46930	CHR714	Os04g47830
		JMJ701	Os03g05680	CHR715	Os04g53720
		JMJ702	Os12g18150	CHR717	Os10g31970
		JMJ705	Os01g67970	CHR721	Os07g44210
	KDM3/JMJD1	JMJ715	Os03g31594	CHR722	Os07g49210
		JMJ716	Os03g22540		
		JMJ718	Os09g22540	CHR724	Os07g44800
		JMJ719	Os02g01940	CHR725	Os08g08220
		JMJ720	Os02g58210	CHR726	Os07g40730
	JmjC domain only	JMJ709	Os01g36630	CHR730	Os03g06920
		JMJ711	Os03g27250	CHR731	Os07g32730
		JMJ710	Os11g36450	CHR732	Os03g22900
		JMJ717	Os08g39810	CHR733	Os02g52510
		JMJ713	Os01g56640	CHR735	Os04g09800
		JMJ714	Os09g31050	CHR736	Os07g25390
		JMJ712	Os09g31380	CHR737	Os06g14440
Histone demethylase (LSD1)		HDMA701	Os04g47270	CHR739	Os07g48270
		HDMA702	Os02g51880	CHR740	Os02g43460
		HDMA703	Os08g04780	CHR742	Os05g32610
		HDMA704	Os10g38850	CHR743	Os08g14610
				CHR745	Os01g44990

acetylation leads to chromatin compaction and gene repression [17] (Fig. 9.2). The dynamic modulation of histone acetylation in plants has been shown to be important for plant gene expression in responding to environmental conditions including light, temperature, biotic, and abiotic stresses [18, 19]. In rice, acetylation of H3 lysine 9 (H3K9) and H4 lysine 12 (H4K12) is elevated in genes located in euchromatic regions [20], suggesting that these markers are associated with active genes. Dynamic and reversible changes in histone H3 acetylation occur at submergence-inducible genes in rice [21]. Recent results have revealed a function of histone acetylation in circadian regulation of rice gene expression [22].

Histone acetylation homeostasis is regulated by antagonistic actions of histone acetyltransferases (HAT) and histone deacetylases (HDAC) (Fig. 9.2). Although HAT function in rice has not been reported, several rice HDAC genes have been studied [23]. The rice genome contains at least 19 HDAC genes belonging to three classes [24]. Among them, two have primary homology to yeast HDAC groups: RPD3 (Reduce Potassium Dependency 3), and SIR2 (Silent mating-type Information Regulation 2). The third group known as the HD2 class is only found in plants [25]. Expression and functional studies suggest that individual rice HDAC genes have specific development functions that may be divergent from the *Arabidopsis* homologues. Expression of rice HDAC genes shows tissue/organ-specificity. Most of the HDAC genes are responsive to drought or salt stresses and some of them display diurnal expression. Over-expression of *OsHDAC1* (also called *HDA702*), a RPD3 class member, leads to increased growth rate and altered architecture in transgenic rice [26, 27]. *OsHDAC1* deacetylates histone H3 lysine 9 (H3K9), lysine 14 (H3K14) and lysine 18 (H3K18) and histone H4 lysine 5 (H4K5), lysine 12 (H4K12) and lysine 16 (H4K16). However, over-expression of several other rice RPD3 members does not produce any visible phenotypes. In contrast, down-regulation of a few RPD3 members causes different developmental defects [23].

SIR2 proteins are NAD+-dependent HDACs, some of which have been found to be involved in metabolic regulation and in increasing lifespan in yeast and animals [28]. In rice or *Arabidopsis* only two *SIR2* genes have been identified [24]. Because the expression pattern of the two rice genes (*SRT701* and *SRT702*) and the subcellular localization of the proteins are different [23, 29, 30], the two genes are likely to have distinct functions. Down-regulation of *SRT701* by RNAi induces H3K9 acetylation, but reduces H3K9 dimethylation (see below for histone methylation) on many loci including transposable elements [30]. Transcription of many transposable elements and some of the hypersensitive response-related genes is activated in the RNAi plants, indicating that in wild-type rice plants transposons and cell death-related genes might be amongst the primary targets of SRT701, suggesting that SRT701-mediated histone deacetylation is an important component for transposon repression in rice.

Rice *HDT701* (*OsHDT1*) belongs to the plant-specific HD2 class of HDACs. Its expression displays a circadian rhythm [22]. Over-expression or down-regulation of the gene does not affect plant growth in an elite hybrid rice parent but the over-expression leads to early flowering of the hybrid under long day conditions [22]. Increased OsHDT1 levels repress the long day flowering repressors *OsGI* and *Hd1* whose expression is increased in the hybrid (a so-called "nonadditive" effect), likely due to increased acetylation levels over the genes. *OsHDT1* over-expression promotes histone H4 deacetylation over *OsGI* and *Hd1* during their peak expression phases in the hybrid and has an effect on nonadditive expression of many other genes in the hybrid [22]. It is possible that OsHDT1 is involved in epigenetic control of parental genome interaction for differential gene expression.

2.2 Histone Methylation and Histone Lysine Methyltransferases in Rice

Histone lysine methylation is an important epigenetic modification with both activating and repressive roles in gene expression [31].

Histone lysine residues can be mono- di- or trimethylated. For instance, H3K9 can be found at mono (H3K9me1)-, di (H3K9me2)-, or trimethylated (H3K9me3) state. Each methyl state may have a different function for genome activity. In plants, H3K9me2 is almost exclusively associated with heterochromatin regions (Fig. 9.2), while H3K9me3 is associated with genes. Trimethylated H3 lysine 27 (H3K27me3) is negatively correlated with gene expression, whereas trimethylated H3 lysine 4 (H3K4me3) and lysine 36 (H3K36me3) are associated with active genes [32] (Figs. 9.1 and 9.2).

2.2.1 H3K4 Methylation

In *Arabidopsis*, H3K4 methylation is found over about two-thirds of genes and is underrepresented in repeats and transposon-rich regions of the genome [33]. While H3K4 monomethylation (H3K4me1) and dimethylation (H3K4me2) are associated with both active and inactive genes, H3K4me3 is mostly correlated with active genes. H3K4me3 and H3K4me2 are detected mostly at the promoter and the 5′ end of genes (Fig. 9.1). In rice about half of the protein-coding genes have di- and/or trimethylated H3K4 based on the analysis of two chromosomes [34]. Rice genes with predominant H3K4me3 methylation are actively transcribed, whereas genes with predominant H3K4me2 methylation are transcribed at moderate levels [9, 34]. It has been shown that H3K4me3 increases over inducible genes in plants upon application of inductive signals [21].

Enzymes involved in histone methylation usually contain a motif called a SET domain, which is named after 3 *Drosophila* genes: Su(var)3-9, Enhancer of zeste (E(Z)), and Trithorax, the mutation of which either enhance or suppress epigenetic mutations [35] (Fig. 9.2). A large number of SET-domain genes are identified in rice and *Arabidopsis* genomes (Table 9.1). Trithorax proteins are a group of methyltransferases for H3K4 methylation. *Arabidopsis* TRITHORAX-RELATED1, 2 (ATX1, 2) and ATX-Related7 (ATXR7) have been shown to be involved in H3K4 methylation [36–38]. Other SET-domain proteins (SDG2) have been recently shown to be also involved in H3K4 methylation [39]. Rice homologues of ATX have been identified [40] (Table 9.1), while their function remains to be characterized.

2.2.2 H3K9 Methylation

Methylation of H3K9 is important for chromatin structure and gene regulation. H3K9me2 is found to be enriched in heterochromatic repetitive sequence regions, while H3K9me3 is distributed in the 5′ end of genes in euchromatic regions and is considered as a "mild" activating mark of gene transcription [41, 42] (Figs. 9.1 and 9.2). Drosophila Su(var)3-9 protein was the first identified histone lysine methyltransferase specific for H3K9 [43]. Plant genomes encode many *SUVH* genes [44]. *Arabidopsis SUVH*, also known as *KYP* (*KRYPTONITE*), and *SUVH5* and *SUVH6* encode activities of H3K9 mono- and dimethyltransferases [45–47]. The rice genome encodes 12 *SUVH* genes, among which *SDG714* is found to be involved in H3K9me2 and DNA methylation of *Tos17*, a *copia*-like retrotransposon [48]. A systematic study of rice *SUVH* genes indicated that different members display distinct function in histone H3K9 methylation, DNA methylation, and transposon silencing [44].

2.2.3 H3K27 Methylation

All three methylated states of H3 lysine 27 are generally associated with repressive chromatin. In *Arabidopsis*, H3K27me3 is associated with about 10 % of annotated genes that are expressed at low levels or repressed in a tissue-specific manner [42, 49], whereas H3K27me1 is mostly associated with silent transposable elements and repetitive sequences [50]. H3K27me3 is distributed all over the gene body region (Fig. 9.1). About a similar percentage of genes are marked by H3K27me3 in rice [9], which are mostly repressed genes [50a].

E(Z) homologues which are components of polycomb group (PcG) complexes are responsible for trimethylation of H3K27 (Fig. 9.2). Several homologues of E(Z) in *Arabidopsis* have been shown to behave as essential regulators of plant

developmental transitions by maintaining repression of key developmental regulatory genes [51]. Homologues of E(Z) and other PcG genes have been identified in rice [52]. Loss-of-function of rice E(Z) genes does not lead to similar defects found in mutants of *Arabidopsis* E(Z) homologues [52], suggesting that developmental function of these genes may not be conserved between different plant species. In *Arabidopsis*, two other ATX-Related genes, ATXR5 and ATXR6, are shown to be responsible for H3K27 monomethylation over repetitive sequences [50] (Fig. 9.2). The rice genome contains a higher proportion of repetitive sequences. Whether homologues of ATXR5 and ATXR6 or additional proteins are involved in H3K27me1 remains to be determined.

2.3 Histone Demethylases

Repression of active genes implies removal of methyl groups from H3K4me3, while activation of repressed genes may require demethylation of H3K27me3 (Fig. 9.2). Histone methylation is reversed by histone demethylases. LSD1 (Lysine specific demethylase 1) was the first histone demethylase to be identified to demethylate H3K4me1 and H3K4me2, in addition to H3K9me1 and H3K9me2 [53]. Four *LSD1* homologues are found in rice and *Arabidopsis*. *Arabidopsis LSD1* genes have been shown to be involved in flowering time regulation [54]. The function of rice LSD1 has not yet been determined. In addition, Jumonji C (jmjC) domain-containing proteins are found to function also as histone demethylases by removing di- and trimethyl groups [55, 56]. More than 20 jmjC protein genes are identified in rice and *Arabidopsis* [57, 58]. Two *Arabidopsis* jmjC proteins, JMJ14 and JMJ15 are shown to possess specific demethylase activity to reverse di- and trimethylated H3K4 [32, 59, 60]. Closely related homologues in rice have also been shown to demethylate H3K4 [60a].

Rice *JMJ706* (Os10g42690) and *Arabidopsis Increase in Bonsai Methylation1* (*IBM1*/*JMJ25*, At3g07610) have activities to remove methyl groups of di- and trimethylated H3K9 in vitro and/or in vivo [58, 61]. Mutations in the two genes produce severe developmental defects, suggesting that histone H3K9 demethylation is essential for normal plant development. Mutations in rice *JMJ706* affect floral organ number and seed development and lead to an increase of H3K9me2/3 [58]. In addition, the phenotype of rice *jmj706* mutants can be partially suppressed by RNAi of a few rice *SUVH* genes [44], indicating that SUVH proteins may form antagonistic couplets with JMJ706 to regulate the homeostasis of H3K9 methylation. It was recently shown that the *Arabidopsis REF6* gene that is closely related to rice *JMJ705* and *JMJ706* is involved in demethylation of H3K27me3 [62]. It remains to be determined whether these rice proteins also have a demethylase activity of H3K27me3.

3 Recognition of Histone Modifications

Histone modification modules are recognized by chromatin proteins that have activities to remodel chromatin structure to regulate gene transcription or induce heterochromatin formation (Fig. 9.2). Acetylated histone lysine residues (e.g., H3K14ac) are bound by bromodomain-containing proteins, such as histone acetyltransferase GCN5 [63]. The different methylated histone lysine residues are recognized by different chromatin protein modules including chromodomains and PHD (Plant HomeoDomain) fingers. For instance, in animal cells the chromodomain of Heterchromatin Protein1 (HP1) binds to H3K9me2 [64], while the chromodomain of the polycomb protein is associated with H3K27me3 [65]. However, in *Arabidopsis*, the chromodomain of LHP1 (LIKE HETEROCHROMATIN PROTEIN 1) interacts with H3K27me3 [42]. A subset of PHD finger-containing proteins are able to interact with H3K4me3 [66]. In addition, chromodomains can also recognize methylated H3K4. For instance, mammalian chromodomain protein CHD1 binds

to H3K4me3 to regulate gene activation [67]. A rice chromodomain protein CHD3 has been shown to be able to interact with both H3K4me2 and H3K27me3 [50a].

4 Interplay Between Chromatin Modifications

Regulation of chromatin remodeling processes involves functional interactions between multiple chromatin modifications (Fig. 9.2). For instance, H3K4me3 is positively associated with H3K9ac. H3K4me3 and H3K9ac are active marks, and represent a chromatin signature of active genes in plants [68]. In addition, H3K9ac is shown to be antagonistic to H3K9me2 in rice. Down-regulation of the HDAC gene *SRT701* not only increases H3K9ac but also reduces H3K9me2 leading to transcriptional activation of many transposable element-related genes in rice [30]. It is likely that deacetylation promotes H3K9 methylation required for transposable element silencing in rice. Genome-wide analysis indicates that H3K4me3 and H3K27me3 are mutually repulsive and antagonistic marks in plants [33, 68]. In rice, relatively few genes are co-modified by both marks [50a]. Interplay between the two histone modification marks is likely to play an important role in gene expression in rice [9]. This may imply physical or functional interactions between histone demethylases and other histone modification complexes as revealed in animal cells [31]. It remains to be known whether similar protein complexes exist in plant cells to coordinate methylation/demethylation of H3K4 and H3K27.

DNA methylation plays critical roles in epigenetic processes and is associated with histone methylation. DNA methylation requires unmethylated H3K4 nucleosomes in mammalian cells [69], while H3K27me3 seems to be antagonistic to DNA methylation in plants [70]. De novo DNA methylation can be triggered not only by small RNA but also by H3K9me2. In *Arabidopsis*, dimethylation of H3K9 by KYP is required for maintenance of CHG methylation by CMT3 [71]. Conversely, the H3K9 demethylase *IBM1*/*JMJ25* has a function to protect active genes from H3K9me2 and CHG methylation [72]. However, mutations of the rice homologue *JMJ706* that affect overall H3K9me2 did not affect DNA methylation in gene bodies (unpublished data), suggesting that JMJ706 may be functionally distinct from IBM1. By contrast, loss-of-function of a H3K4 demethylase gene *JMJ14* leads to loss of non-CG methylation at loci targeted by RNAi-directed DNA Methylation (RdDM) in *Arabidopsis* [73]. Therefore histone demethylation on different lysine residues may have distinct roles in DNA methylation regulation [57]. It remains to be known whether similar interactions are conserved in rice that has a larger amount of repetitive sequences and transposable elements than *Arabidopsis*.

5 Epigeneomic Variations, Inheritance and Epialleles

Closely related species may develop differences in their epigenetic systems during adaptation to different environmental niches. Studies of natural variation of DNA methylation in a number of other plant species have suggested that epigenetic variation among individuals with similar genotype can lead to phenotypic variation in response to varying environmental conditions [74]. Epigenetic adaptive responses to environmental cues can be transmitted to future generations [75, 76].

It has been shown that DNA methylation in genes is extremely polymorphic among 96 natural accessions of *Arabidopsis* [77]. More recent studies by examining spontaneously occurred variation in DNA methylation in *Arabidopsis* plants propagated by single seed descent for 30 generations have revealed that transgenerational changes in cytosine methylation occur at a high frequency [78, 79]. Transgenerational variation in DNA methylation that adversely affects gene expression may generate new epigenetic alleles (epialleles) leading to phenotypic variation without DNA sequence change. DNA methylation-induced silencing of protein-coding genes gives rise to epialleles that can be inherited through

meiosis [80]. Two examples of meiotically heritable epialleles resulting in morphological variation are the *peloric* (in *Linaria vulgaris*) and *colorless non-ripening* (in *Solanum lycopersicum*) loci which are spontaneous epigenetic silencing events [81, 82]. In rice, the epiallele of *DWARF1* (*D1*), *Epi-d1*, causes a metastable dwarf phenotype [83]. The silenced state is correlated with repressive histone and DNA methylation marks in the *D1* promoter region. It has been recently shown that the expression level of *OsSPL14* (*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE14*) is important to regulate panicle branching and grain yield in rice [83]. Differences in DNA methylation and histone modifications on the *OsSPL14* locus are likely to be responsible for the expression difference of the gene between two *japonica* rice varieties that differ in grain numbers per panicle. This case demonstrates that epigenetic mutations may be an important source for variation of important agronomic traits in rice.

6 Perspectives

In the long history of rice evolution, domestication and selection, epigenomic variations may have been generated and may have contributed to phenotypic differences and variations in complex traits among different species, subspecies, and cultivars. In addition, epigenomic variation among individuals with similar genotypes can drive phenotypic variation in response to varying physical, biotic, and abiotic environments. Many morphological and adaptive phenotypes may be dependent on different epialleles. Therefore, investigating rice and other *Oryza* epigenomes will be important to identify specific epigenetic marks and epialleles involved in important agronomic and adaptive traits. Functional characterization of rice chromatin modification regulators (writers, erasers, and readers) has just started and remains to be an important research field to understand the mechanism of establishment, maintenance, recognition and inheritance, or erasure of rice epigenomes. In fact, understanding how established epigenomic marks corresponding to specialized plant cell types or responding to induction by specific environmental cues can be memorized during subsequent cell divisions and inherited to next generations represents an essential research aspect of epigenetics in the future.

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Noncoding Regulatory RNAs

10

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Noncoding RNAs (ncRNAs) refer to RNA molecules that are not translated into proteins but possess intricate regulatory and structural functions. Various classes of ncRNAs have been identified in eukaryotes, including microRNAs (miRNAs), small interfering RNAs (siRNAs), transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs), antisense RNAs, and other long noncoding RNAs (lncRNAs), etc. These ncRNAs play diverse roles in many biological processes mainly through transcriptional and posttranscriptional regulations [74]. The importance of ncRNAs has been implicated from the evolutionary point of view that the organismal complexity of eukaryotes correlates directly with the proportion of noncoding sequences in their genomes [100]. For example, during vertebrate evolution, there was a dramatic expansion of ncRNA genes which added extra layers of regulatory complexity to adapt the fitness of further developed organisms. Increasing lines of evidence have revealed that plant ncRNAs play crucial roles in the regulation of plant development, especially for some important agronomic traits in crops. This chapter summarizes current advances in the understanding of biogenesis, regulatory targets, and functions of rice ncRNAs.

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1 Small RNAs in Rice

Eukaryotic endogenous small RNAs are one of the major types of ncRNAs and are usually ~20–30 nucleotides (nt) in length. In higher plants, small RNAs comprise two types, namely miRNAs and siRNAs, which negatively regulate gene expression at either the transcriptional or posttranscriptional levels and have been shown to be involved in developmental patterning, maintenance of genome integrity, and responses to abiotic and biotic stresses [9]. The biogenesis and functions of plant small RNAs have been well documented in *Arabidopsis*, a dicot model system [8, 13, 72]. Although many advances in the study small RNAs in rice used the knowledge of *Arabidopsis* as a reference, current progress strongly indicates that a more complex small RNA network exists in rice as compared to that in *Arabidopsis*, and they play wider or more important regulatory roles.

2 Key Components Involved in Small RNA Biogenesis in Higher Plants

The biogenesis of miRNAs and siRNAs in higher plants share two families of proteins: DICER-LIKE (DCL) enzymes to process small RNAs from precursors, and ARGONAUTE (AGO) proteins to perform silencing effector functions in RNA-induced silencing complexes (RISC). DCL is a multi-domain RNaseIII enzyme, which

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is characterized as a double stranded RNA (dsRNA)-specific endonuclease, and typically contains six conserved domains including a DExD-helicase, a helicase-C, a Duf283, a PAZ, two RNase III and a dsRNA-binding domain [71]. The PAZ and RNaseIII domains play central roles in processing of small RNAs, wherein PAZ domains bind the duplex ends of double-strand shaped RNA molecules or RNA duplexes with short 3′ overhangs, and each of the two RNase III domain cleaves one of two strands of dsRNA precursors [70, 123]. AGO proteins contain four domains referred to as PAZ, PIWI, N terminal, and Mid-domains. The functional PAZ domain binds to the 3′ end of the guide strand, and the PIWI domain has an RNase H-like fold and catalyzes endonucleolytic cleavage of target RNAs [106, 108]. Multiple DCLs and AGOs are encoded in higher plant genomes, such as 4 DCLs and 10 AGOs in *Arabidopsis* and 8 DCLs and 19 AGOs in rice [41]. In both *Arabidopsis* and rice, phylogenetic analysis showed that members of both DCL and AGO families can be divided into four subgroups, namely DCL1–DCL4 for DCLs and AGO1, AGO4, ZIPPY, MEL1 for AGOs [41]. Mounting evidence has established that multiple DCLs and AGOs govern functional specialization in the biogenesis of small RNAs. The enlarged gene families of DCLs and AGOs in rice suggest that more elaborate or wider small RNA biogenesis mechanism might exist in rice as compared to *Arabidopsis*.

3 miRNAs in Rice

miRNAs are 20–24 nt in length and are generated from the stem-loop regions of single-stranded RNA precursors. They negatively regulate gene expression by binding to target mRNAs to either induce mRNA degradation or repress protein translation. Since the first miRNA miR-lin4 from *Caenorhabditis elegans* was identified in 1993, numerous miRNAs have been found in many eukaryotes and they appear to participate in the regulation of almost all essential biological processes. Over the past several years, next generation sequencing technologies and computational prediction approaches have accelerated the identification of less abundant miRNAs in various species. Currently, 708 mature miRNAs encoded by 591 precursors of *Oryza sativa* were collected in the miRBase 19.0 (http://microrna.sanger.ac.uk/).

4 miRNA Biogenesis in Rice

Progresses in *Arabidopsis* miRNA research have revealed a relatively intact miRNA biogenesis pathway [103]. This pathway appears to be conserved in rice, despite the fact that functions of some protein orthologs remain elusive (Fig. 10.1a). In the miRNA biogenesis process, the primary miRNA precursors (pri-miRNAs), transcribed by RNA polymerase II (PolII) [53], can self-fold into stem-loop shaped structures. Subsequently, the RNA binding protein DAWDLE (DDL) binds to pri-miRNAs and facilitates DCL1 to access or recognize pri-miRNAs [88, 122]. The DCL1, assisted by nuclear body (referring as D-body)-containing proteins including a dsRNA-binding protein HYPONASTIC LEAVES (HYL1) and a Zinc finger protein SERRATE (SE), orchestrates two consecutive processes: processing pri-miRNAs to pre-miRNAs and pre-miRNAs to mature miRNAs [17, 48, 50, 63]. The mature miRNAs are then methylated at their 3′ ends by HUA ENHANCER (HEN1) to avoid uridylation and degradation by the SMALL RNA DEGRADING NUCLEASE (SDN) class of exonuclease [1, 56, 88, 121]. Subsequently, mature miRNA are sorted into the AGO1-, AGO7-, or AGO10-containing effector complex RISC, to perform gene regulation at transcriptional or posttranscriptional levels [32, 79, 115, 129].

In rice, loss-of-function mutations disrupting miRNA biogenesis factors result in similar and pleiotropic developmental defects (Fig. 10.1b). Strong transgenic lines knocking down *DCL1* or *AGO1* showed dwarfism with rolled leaves and tortuous shoots, as well as seedling stage developmental arrest; Weak transformants also displayed severe developmental defects including mild dwarfism, narrow and rolled leaves [63, 115]. Phenotypes of HEN1 ortholog mutants, referred to as *WAVY LEAF1* (*waf1*), were also reminiscent

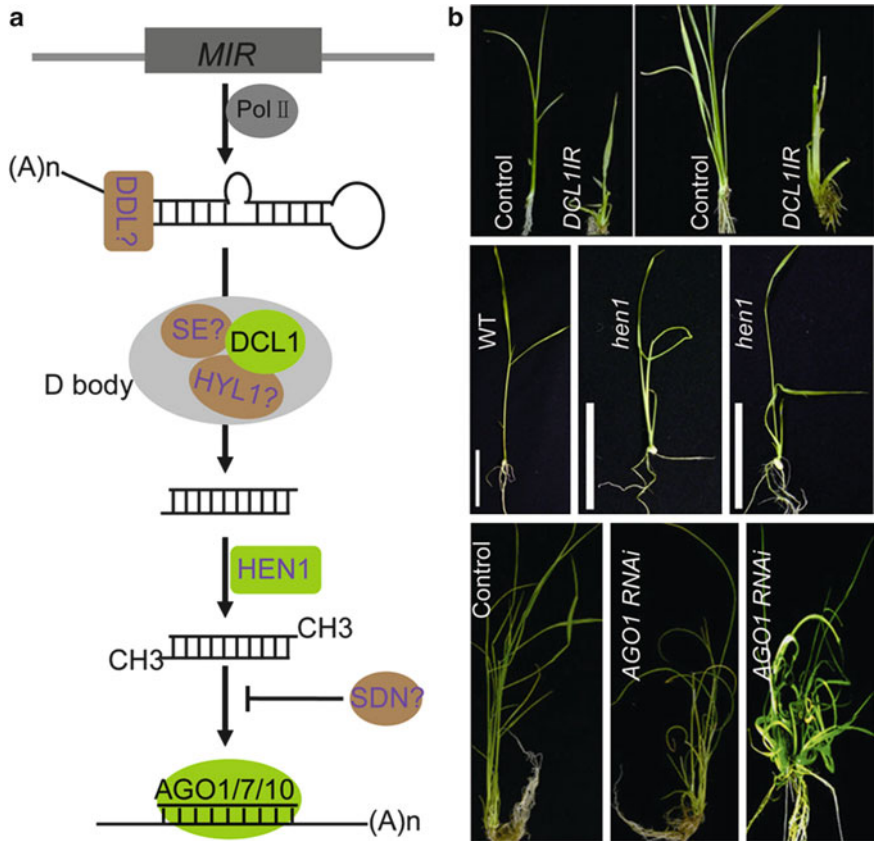

Fig. 10.1 Biogenesis pathway and functional illustration of rice miRNAs. (**a**) A model for miRNA biogenesis in rice. (**b**) Phenotypes of RNAi lines of *DCL1*, *AGO1,* and *hen1* mutant of rice. Images reprinted with permission from [63] for *DCL1*, from [1] for *hen1* and from [115] for AGO1

of *DCL1* or *AGO1s* knock-down lines, with wavy leaves and early death after germination [1]. These severe developmental defects in loss-of-function mutants of rice miRNA biogenesis pathway genes suggest the critical roles of miRNAs in the regulation of rice development.

5 Identification of miRNA Targets in Rice

miRNAs confer their roles mainly in posttranscriptional gene regulation, in which the mature miRNAs act as guide molecules to impel AGO1/7/10-mediate cleavage or translation inhibition for target mRNAs. RISC-mediated cleavage is the major regulatory machinery for plant miRNAs, owning to the perfect or nearly perfect sequence of complementary miRNAs with their target mRNAs. Based on this as well as the slicing property: cutting precisely between the 10th and 11th nt from the 5′ end of the miRNA, the bioinformatics prediction of plant miRNA targets is the first method to screen the miRNA targets. Then, experimental validation is achieved through detection of slicing products by northern blot or by 5′ rapid amplification of cDNA ends (RACE). Recently, degradome sequencing technology, a genome-wide method which characterizes the remnants of small RNA-mediated RNA cleavage by sequencing the 5′ end of uncapped RNAs within cells, has enabled genome-wide experimental identification of miRNA targets in plants [2, 20]. By combining degradome sequencing

Table 10.1 Conserved miRNAs and their targets in *rice* (*Oryza sativa* L. *ssp*.)

miRNA family	Target genes
osa-miR156	Os01g69830[b,c]; Os02g04680[a,b,c]; Os02g07780[a,c]; Os04g46580[b]; Os06g45310[a,b,c]; Os06g49010[a,b,c]; Os07g32170[a,b,c]; Os08g39890[b,c,(d)]; Os08g41940[b]; Os09g31438[b,c]; Os09g32944 [b,c]; Os11g30370[a,b,c]; Os05g48800[c]; Os07g36500[c]
osa-miR159	Os01g59660[a,(b,c,e)]; Os06g40330[a,b,c]; Os01g11430[a,b]; Os05g42240[b]; Os09g36650[b]; Os10g05230[a,b]
osa-miR160	Os02g41800[a,(b),c]; Os04g43910[a,(b),c]; Os04g59430[a,(b)]; Os06g47150[a,(b),c]; Os10g33940[a,(b),c]
osa-miR162	Os03g02970[(b),c]
osa-miR164	Os02g36880[a,c]; Os04g38720[a,c]; Os06g23650[b]; Os06g46270[a,c]; Os08g10080[c]; Os12g41680[a,(b),c]; Os03g50040[c]; Os05g39650[c]; Os12g05260[a,b]
osa-miR166	Os03g01890[a,(b),c]; Os03g43930[b,c,(f)]; Os10g33960[a,b,c]; Os12g41860[(b),c]; Os01g08520[b]; Os04g48290[a]
osa-miR167	Os02g06910[c]; Os04g57610[a,(b,f,g),c]; Os06g46410[c]; Os12g41950[c]; Os06g03830[(c)]; Os07g33790[c];Os07g29820[c]
osa-miR168	Os02g45070[(b),c]; Os02g58490[c,(f)]; Os04g47870[(b),c]; Os06g51310[c]
osa-miR169	Os02g53620[a,(b,f),c]; Os03g07880[a,b,(c)]; Os03g29760[a,b,c,(h)]; Os03g44540[b,(c)]; Os03g48970[b,c]; Os07g06470[a,b,c]; Os07g41720[a,b,c]; Os12g42400[a,b,c,(f)]
osa-miR171	Os02g44360[a,b,c]; Os02g44370[a,b,c]; Os04g46860[a,(b),c]; Os10g40390[b]; Os04g39864[(c)]
osa-miR172	Os03g60430[(b),c]; Os04g55560[a,b,c,(i)]; Os05g03040[a,b,c,(f)]; Os06g43220[b,(i)]; Os07g13170[a,b,c,(i)]; Os01g04550[c]; Os04g36054[b]; Os08g39630[b,c]
osa-miR319	Os01g55100[(b)]; Os03g57190[a,b]; Os07g05720[a,(b),c]; Os12g42190[(b)]
osa-miR390	TAS3a[(b),c]; TAS3b[(b),c]; TAS3c[(b),c]; Os02g10100[(b,j)]
osa-miR393	Os04g32460[a,b,c]; Os05g05800[a,(b),c]; Os03g36080[c]; Os04g58734[(c)]
osa-miR394	Os01g69940[a,(b),c]; Os05g51150[b]
osa-miR395	Os10g35870[b]; Os06g05160[c]
osa-miR396	Os02g45570[b,c]; Os02g47280[a,b,c]; Os02g53690[b,c]; Os03g47140[b,c]; Os03g51970[b,c]; Os04g48510[b,c]; Os04g51190[b,c]; Os06g02560[a,b,c]; Os06g10310[b]; Os11g35030[a,b,(c)]; Os12g29980[b]; Os01g32750[c]; Os06g29430[c]; Os01g44230[b]; Os05g45220[b]; Os12g05000[b]
osa-miR397	Os11g48060[c]
osa-miR398	Os03g22810[c]; Os04g48410[(c)]; Os07g46990[a,(b),c]
osa-miR399	Os05g48390[b,c]
osa-miR408	Os02g52180[b,c]; Os03g50140[(b)]; Os04g46130[b,c]; Os06g11490[b,c]; Os08g37670[(b),c]

[a]Degradome sequencing validation by Wu et al. [115]
[b]Degradome sequencing validation by Zhou et al. [128]
[c]Degradome sequencing validation by Li et al. [60]
( ), 5′ Race validation by [d][36]; [e][68]; [f][64]; [g][120]; [h][124]; [i][131]; and [j][95]

with 5′-RACE validation, a systemic characterization of rice miRNA targets has been performed in Nipponbare (*Oryza sativa ssp*. *japonica*) seedlings [60] and seedlings/panicles [115], and panicles of *93–11* (*Oryza sativa ssp*. *indica*) [128]. Between Arabidopsis and rice, the target genes of 21 conserved and 45 non-conserved miRNA families have been identified in total (Tables 10.1 and 10.2) [5]. The majority of miRNA families have multiple target genes, suggesting their diverse regulatory roles. In addition, most target genes of conserved miRNAs were transcription factors and captured in at least two libraries from different tissues or subspecies of rice, suggesting their broad expression and essential functions (Table 10.1). In contrast, many targets of non-conserved miRNAs were only identified in one library (Table 10.2), which may contribute to tissue, organ, or subspecies regulation between rice-specific miRNAs and their target genes.

6 Regulatory Roles of miRNAs in Rice Development

In rice, the functions of several miRNAs have been characterized using genetic approaches including over-expression of miRNAs or

Table 10.2 Non-conserved miRNAs and their targets in rice (*Oryza sativa* L. *ssp*.)

miRNA family	Target genes
osa-miR413	Os03g63380[b]
osa-miR414	Os01g03410[b]; Os01g08560[b]; Os01g16670[b]; Os01g48180[b]; Os01g70330[b]; Os03g58590[b]; Os03g59760[b];Os03g62620[b]; Os04g13880[b]; Os04g37940[b]; Os05g51490[b]; Os05g51830[b]; Os06g33520[b]; Os06g50880[b]; Os07g40450[b]; Os08g09270[b]; Os08g33370[b]; Os01g55880[c]; Os08g07540[c]; Os09g38400[b]; Os10g16520[b]; Os11g14220[b]
osa-miR415	Os01g73160[b];Os04g52050[b]
osa-miR419	Os05g06280[b]
osa-miR437	Os02g18080[b]
osa-miR441	Os09g09820[b]
osa-miR444	Os04g38780[a,c,(b,k)]; Os08g33488[c]; Os02g36924[a,c,(k)]; Os02g49840[a,c,(k)]; Os05g47560[a]; Os02g49090[c]; Os03g63750[(c)]; Os05g08410[c]; Os08g33479[c]; Os02g13420[(c)]; Os03g54084[c]; Os08g06510[a,(b)]; Os03g32499[b]
osa-miR446	Os02g29140[b,c]; Os03g52010[b]; Os04g38450[b,c]; Os06g19990[b,c]; Os08g44850[b]; Os09g09820[b]; Os10g26720[b,c]
osa-miR528	Os06g06050[a,(b,c)]; Os06g11310[b,c]; Os06g37150[a,(b),c]; Os07g38290[(b)]; Os08g04310[(b)]
osa-miR529	Os01g69830[b]; Os08g39890[(b)]; Os09g31438[b]; Os09g32944[b]; Os06g47780[c]; Os06g04270[c]
osa-miR530	Os02g14990[a]
osa-miR806	Os02g34950[b]; Os02g43370[b]; Os03g09230[b]; Os12g40560[b]
osa-miR808	Os02g44990[b]; Os02g45650[b]; Os03g50070[b]; Os04g02640[b]; Os04g32610[b]; Os06g47850[b]; Os08g06500[b]; Os08g19114[b]; Os08g36840[b]; Os08g40440[b]; Os10g39970[c]; Os12g40920[c]
osa-miR809	Os04g58070[b]; Os06g33210[c]; Os10g40540[c]; Os02g55480[c]
osa-miR812	Os02g23823[b]; Os03g12620[b]; Os03g22050[b,c]; Os06g38210[b]; Os06g50146[b]; Os11g09260[b]
osa-miR814	Os12g39380[c]
osa-miR818	Os01g63880[b]; Os02g10210[b]; Os03g48010[b]; Os03g49126[b]; Os04g39160[b]; Os04g57154[b]; Os06g11500[b]; Os06g39330[b]; Os08g29760[b]; Os08g41080[b]; Os09g36320[b]
osa-miR819	Os03g31180[b]; Os08g38620[b]; Os09g30140[b]; Os10g39970[b]; Os12g02520[b]
osa-miR820	Os03g02010[b,c,(k,e,m)]
osa-miR827	Os04g48390[b,c,(l,n)]
osa-miR1320	Os05g47550[a]
osa-miR1425	Os10g35240[a,(k,i)]; Os08g01640[c]; Os08g01650[c]; Os08g01870[c]; Os10g35230[c]; Os10g35460[c]; Os10g35640[c,(k)]; Os01g49580[c]; Os01g49614[c]; Os05g40700[c]
osa-miR1426	Os05g30350[c]
osa-miR1428	Os03g17980[a,c,(o)]
osa-miR1431	Os02g16670[c]
osa-miR1436	Os01g63880[b]; Os02g13210[b]; Os02g43560[b]; Os03g15350[b]; Os05g50570[b]; Os06g11500[b]; Os07g03110[b]; Os07g40450[b]; Os09g36320[b]; Os03g48010[c]
osa-miR1439	Os06g50146[b]; Os07g17250[b]; Os09g33690[c]
osa-miR1440	Os03g05200[b]
osa-miR1442	Os02g58670[b,c]
osa-miR1846e	Os06g14060[c]; Os09g07510[c]
osa-miR1847	Os02g34990[c]; Os04g39170[c]
osa-miR1848	Os01g49720[c]
osa-miR1850	Os04g47410[a]; Os01g02360[c]; Os09g32250[c]; Os05g05140[c]
osa-miR1857	Os11g45590[a]; Os01g05790[c]
osa-miR1858	Os03g56060[b]
osa-miR1884	Os01g59720[a,b]; Os11g34910[a]; Os03g10250[a]; Os01g64520[a]; Os03g19380[a,b]; Os02g22610[a,b]
osa-miR2093	Os01g72650[b]; Os11g06370[b]
osa-miR2098	Os03g23050[c]
osa-miR2095	Os04g32340[b]

(continued)

Table 10.2 (continued)

miRNA family	Target genes
osa-miR2101	Os04g42090[b]; Os12g08210[b,c]
osa-miR2102	Os05g37460[c]
osa-miR2103	Os03g52880[c]
osa-miR2015	Os01g14040[c]; Os01g25484[c]
osa-miR2098	Os07g03279[c]; Os07g03368[c]; Os07g03458[c]
osa-miR2868	Os11g14140[c]

[a]Degradome sequencing validation by Wu et al. [115]
[b]Degradome sequencing validation by Zhou et al. [128]
[c]Degradome sequencing validation by Li et al. [60]
( ), 5′ Race validation by [k][65]; [l][49]; [m][116]; [n][25]; [o][130]

miRNA-resistant targets (nontargeted), miRNA target mimicry and isolation of mutants disrupting the interaction of miRNA and their targets.

MiR156 is a conserved miRNA family, which contains 12 members (miR156a–miR156l) in the rice genome and negatively regulates *SOUAMOSA* (*SQUA*) *PROMOTER-BINDING-LIKE* (*SPL*) transcription factors. Among the 19 rice *SPL* genes, 12 were miR156 targets (Table 10.1) [118]. miR156 mainly accumulated in the young shoots and leaves whereas majority of miR156-targeted genes were primarily expressed in young panicles [118], suggesting their negative regulation. Over-expression of miR156b and miR156h resulted in severe dwarfism and increased tiller numbers, reduced panicle size, and late flowering, which suggests that miR156-mediated regulation on *SPLs* might involve in various developmental processes in rice (Fig. 10.2). Recently, subsequent study of leaf and tiller developments revealed that expression of rice miR156 showed a gradual increase during leaf development, and disruption of the miR156 gradient by over-expression of miR156b and miR156h impaired the temporal expression patterns of thousands of genes, most of which might play essential role in regulating leaf and tiller development [117].

Recently, two independent lines of evidence have illustrated the important roles of miR156-mediated regulation of *OsSPL14* (also known as *IDEAL PLANT ARCHITECTURE1* (*IPA1*), and *WEALTHY FARMER'S PANICLE* (*WFP*)) on controlling rice architecture [36, 78]. IPA mutants have low tiller numbers, increased panicle yield, thick and sturdy stems and have been proposed to be effective in increasing grain yield [43]. *OsSPL14*$^{ipa1/IPA1}$ and *OsSPL14*^{Aikawa1} loci isolated from two *japonica* rice varieties of Shaoniejing (SNJ) and Aikawa1, respectively, harbor the same point mutation in *OsSPL14* (*Os08g39890*) which leads to a mismatch between miR156 and *OsSPL14* and perturbs miR156-directed cleavage of *OsSPL14* mRNAs. Therefore, the up-regulated *OsSPL14* transcripts in the reproductive stage promoted panicle branching and resulted in higher grain yield in rice. Direct evidence that the loss of regulation of miR156 increased the IPA traits was derived from the generation of nontargeted *OsSPL14* mutants (*OsSPL14*^{IPA17m}-*GFP*/*pNIP*:*OsSPL14*) in Nipponbare, which changed the miR156 target sites of *OsSPL14* without altering its protein sequence. *OsSPL14*^{IPA17m}-*GFP* showed similar phenotypes as *OsSPL14*^{ipa1}-*GFP*, and both with increased transcript and protein levels of *OsSPL14*. When *OsSPL14*^{ipa1} and *OsSPL14*^{Aikawa1} alleles are introduced into some rice varieties by either transformation or introgression, both transgenic and near-isogenic lines (NILs) showed increased panicle branch number and grain yield, suggesting that *OsSPL14*^{ipa1} and *OsSPL14*^{Aikawa1} alleles have great potential for breeding of elite rice varieties with an ideal plant architecture.

Seven *SPL* genes including *SPL14* were also predicted to have an miR529a-5p target site, which is located at the 4 nucleotides 5′ upstream of the miR156 target sites [31]. The point mutations

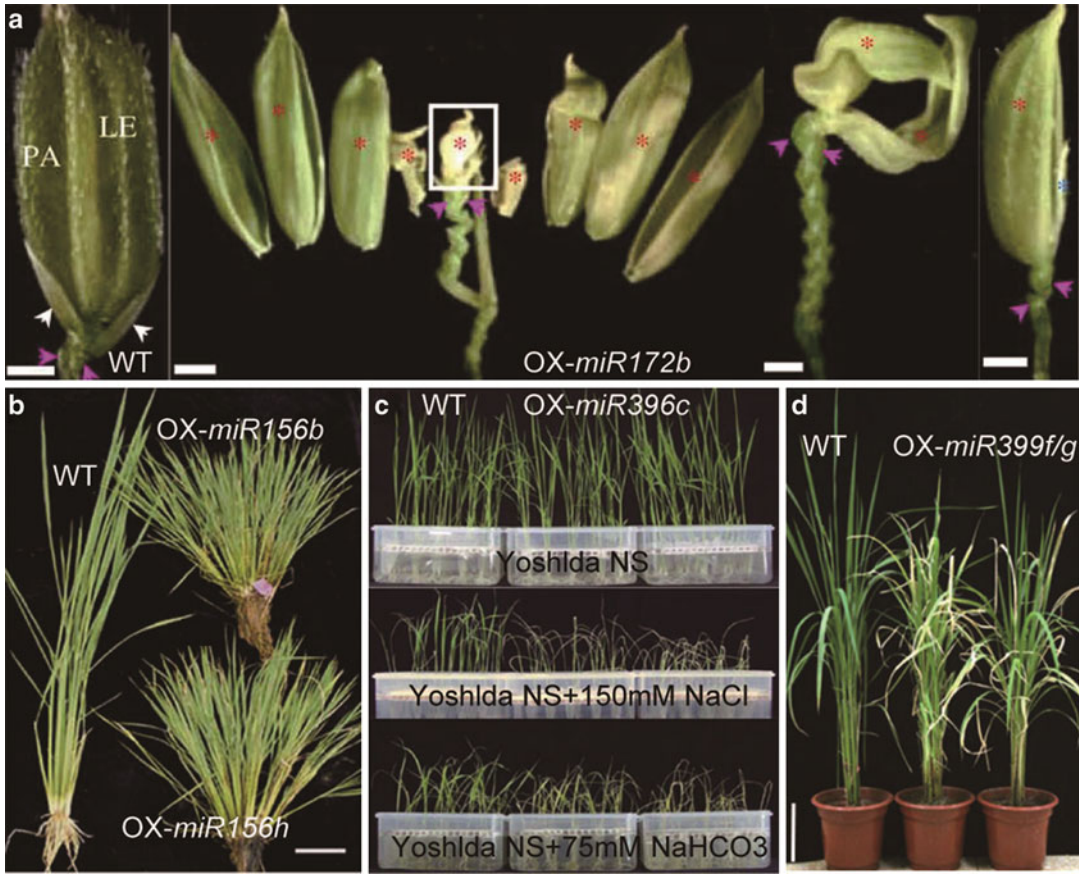

Fig. 10.2 Phenotypes of over-expression of miRNAs in rice. (**a**) Over-expression of miR172b, images reprinted with permission from [131]; (**b**) over-expression of miR156b/h, images reprinted with permission from [118]; (**c**) over-expression of miR396c under the stress of salt and alkali, images reprinted with permission from [18]; (**d**) over-expression of miR399f/g, images reprinted with permission from [28]

of SNJ and Akiwa1 were also found to be located in the target site of miR529a-5p. Comparing the expression of miR156 and miR529a-5p with their mediated cleavages on *SPL14* in different rice tissues indicated that miR156 and miR529 may perform synergic regulations on *SPL14* in different tissues, in which seedling-enriched miR156 and panicle-enriched miR529 mediate primary regulation on *SPL14* in vegetative and reproductive stages, respectively, [31].

MiR172 is another conserved miRNA family that contains four members (miR172a–miR172d) in rice. miR172-directed regulation of APETALA2 (AP2)-like transcription factors in *Arabidopsis* and maize has been shown to regulate specific floral organ identity or the vegetative phase change [4, 12]. Five *AP2*-like genes, namely *Os03g60430*, *Os04g55560*, *Os05g03040*, *Os06g43220,* and *Os07g13170* (*SNB-SUPERNUMERARY BRACT*), have been predicted and validated as the targets of miR172 in rice (Table 10.1) [51, 131]. Over-expressing miR172b in rice results in pleiotropic defects including a delayed transition from spikelet meristem to floral meristem, altered numbers or floral identity, lower fertility and smaller grains [131] (Fig. 10.2). All of these developmental defects in miR172b over-expression plants phenocopies the loss-of-function mutant of a gene encoding a confirmed target of miR172, namely *SNB* [131]. The expressions of some miR172 targets did not decrease in miR172b over-expression plants [131],

suggesting that miR172 regulation of *AP2-like* expression may be primarily through translational inhibition, the same mechanism shown in *Arabidopsis* [12].

In addition to controlling rice development, a growing body of evidence also suggests the importance of miRNAs in physiological and environmental responses. For example, four members of miR399 (a, d, f, and j) were induced by inorganic phosphate (Pi) deprivation to regulate the Pi-signaling network via modulation of *PHO2*, an unusual E2 conjugase gene in *Arabidopsis* [7]. In rice, the putative *PHO2* ortholog (also known as *LEAF TIP NECROSIS 1*, *LTN1*) contains miR399-binding sites. Mutations in the putative miRNA binding site of *LTN1* lead to typical Pi starvation responses, suggesting miR399-directed *PHO2* regulation in rice is conserved [7, 28]. In addition, the phenotypes of *ltn1* are reminiscent of overexpression of miR399f and miR399j, with leaf tip necrosis in mature leaves, dwarfism, less tiller numbers, and low fertility (Fig. 10.2) [28]. Meanwhile, *LTN1* transcripts were down-regulated in miR399-over-expressing plants. All these findings indicate that miR399-directed *LTN1* regulation is involved in multiple Pi starvation responses in rice.

Members of the miR396 and miR393 families were found to be link with salt and alkali stress. Transgenic plants over-expressing miR396c or miR393 exhibited reduced tolerance to salt and alkali, which are consistent with the decreased levels of both miRNAs under treatment of salt and alkali (Fig. 10.2) [18, 19].

7 Tissue-Specific/Preferential or Induced-Expression miRNAs in Rice

In rice, miRNAs with tissue-specific or induced-expression patterns were also identified. For example, many novel miRNAs or specially/preferentially expressed miRNAs have been identified in embryogenic calli [10, 68], the seedlings [59, 97], developing pollen [111], and developing rice grains [119, 130]. A large-scale survey for 222 miRNA expression patterns using deep-sequencing technology revealed that 60 (27 %) miRNAs exhibited a high degree of tissue specificity [31]. Moreover, some miRNAs were identified in responses to salt [33, 90, 124], cold [31, 69], heat [31], drought [33, 125], heavy metal [29], Pi or S starvation [31], H_2O_2 [59], phytohormone [64], and tungro virus infection [90]. These miRNAs might confer specific roles under distinct stress conditions, providing an extra layer of regulation in rice when exposed to unfavorable environments.

8 Evolutionary Analysis of miRNA Regulation in Rice

It has been shown that some plant conserved miRNAs generally modulate important transcription factors, and thus generate many conserved miRNA regulatory modules throughout the plant kingdom [38]. Therefore, strong selection forces might be required to guarantee the low sequence variation or coevolution between miRNAs and their targets, which has been confirmed in human and *Arabidopsis* [15, 91]. A survey for 88 mature miRNA sequences in 33 rice accessions showed higher sequence variations (11 of 88 investigated miRNAs) than that of *Arabidopsis* (2 of 64 miRNAs), yet the levels of sequence divergence of mature miRNA in rice are still much lower than in their flanking regions [107]. In addition, lower nucleotide variation rate was also observed in miRNA target sites of miRNAs as compared to their flanking regions [22]. Together, the observation in rice supports the findings in human and *Arabidopsis* that strong selection forces drive the low variation or co-evolution of both miRNAs and their targets.

Plant miRNA families with multiple members may have evolved from genome duplication events showing dynamic changes when compared among the rice varieties (intra-species of genus *Oryza*) or compared rice with other plants (inter-species of genus *Oryza*). For example, miR395 family members can be classified into four clusters (a–d) and each transcript can function as an independent unit in the cultivated species with AA genome; whereas two clusters (a and c) are absent in other species of genus *Oryza* with BB, CC, EE, FF, and GG genomes [21]. It is also reported that two miR156 members

(b and c) were arranged as tandem repeats in the rice genome and this array is conserved among cereals, but not in *Arabidopsis* since the tandemly duplicated sequences of miR156b/c in *Arabidopsis* was destroyed by multi gene insertion [105]. Therefore, sequence variation has been observed in some multicopy miRNA families between species of genus *Oryza* and other organisms, which may help to increase the diversification and flexibility of miRNA regulation. Furthermore, the expression patterns of the conserved miR160 and miR162 are quite different in rice and *Arabidopsis* although they both harbor the same sequences [34], reflecting species-specific expression diversification of miRNAs.

9 Noncanonical miRNAs in Rice

The miRNAs mentioned above are referring to the canonical type with following features: ~21-nt length; precisely sliced from the stem of a single-strand, stem-loop precursors; and ancillary criteria include conservation among plants, the DCL1 dependence and the defining targets, etc. [75]. In addition to canonical miRNAs, various types of miRNAs with nonstandard characters were also identified in rice, enriching the regulatory functions of rice miRNAs. Natural antisense miRNAs (nat-miRNAs), originated from the antisense strand of *cis*-antisense transcript pairs, are a special type of miRNAs to negatively regulate *cis*-transcripts, which overlap with pri-miRNA transcripts by inducing mRNA cleavage [65]. The 24-nt long miRNAs (lmiRNAs) are another type of noncanonical miRNA, in which the biogenesis of lmiRNAs is processed by DCL3a instead of DCL1, and loaded into AGO4, but not AGO1, to mediate transcriptional modulation by triggering DNA methylation both *in-cis* and *in-trans* [116].

10 Secondary Small RNAs in Rice

Besides directly conferring roles in regulation of development, some plant miRNAs also mediate the production of secondary small RNAs to perform regulatory functions. Conserved plant transacting small interfering RNAs (tasiRNAs) and rice expressed phased small RNAs, whose biogenesis requires miRNAs to initiate, are two paradigms.

TAS3 is a conserved locus in numerous of plant species, and rice utilizes this site to produce tasiRNAs [3]. Current studies indicate that tasiRNA biogenesis is conserved between rice and *Arabidopsis*. Transcripts of *TAS3* containing miR390 targeted sites are cleaved by the miR390-AGO7 complex to produce truncated RNAs [3, 80]. The cleavable single-strand RNAs were further transformed into double stranded molecules by RNA-dependent RNA polymerase 6 (RDR6) and processed, into 21-nt fragments, by DCL4 into tasiRNAs [62, 93]. These small RNAs were methylated by HEN1 and associated with AGO1 to act *in trans* by guiding the cleavage of targeted mRNAs [1]. The conserved tasiRNAs, namely tasiR-ARFs, are produced from the 5′ D6 and 5′ D7 phased loci, which counted from the 5′ sequence of miR390 directed cleavage point at rice *TAS3* [62]. Five members belonging to *AUXIN RESPONSE FACTORS*, known as *ARF2* and *ETTIN/ARF3*, were identified as the targets of rice tasiR-ARFs [60, 62]. Mutants of *RDR6* (*SHL2*), *AGO7* (*SHL4*), and *DCL4* (*SHO1*), in which tasiRNAs biogenesis is interrupted, cause complete deletion or abnormal formation of the shoot apical meristem (SAM) [80]. Plants with impaired *DCL4* and *RDR6* also showed abaxial-adaxial polarity defects such as thread-like lemma and disturbed anthers [62, 93, 102]. All of these mutant defects can be attributed to miR166-mediated down-regulation of *HD-ZIPIII* and tasiR-ARF-mediated up-regulation of *ARFs*. Loss-of-regulation of tasiR-ARFs by expression of nontargeted *ARF3* mutants were shown to mimic the mutant phenotypes by disrupting the biogenesis of tasiRNAs [62, 80, 93]. The balance established by tasiR-ARFs and miR166 is essential for proper generation of organ polarity, which is further confirmed by over-expressing Osta-siR2141 (tasiR-ARFs) [104]. Transgenic plants with over-expressing Osta-siR2141 showed irregular SAM, vegetative stage growth retardation, disturbed vascular bundle, and adaxialization in organ polarity, accompanied by a decrease

of miR166 and an increase of *HD-ZIPIII* [104]. Together, by combining with evidence gathered from *Arabidopsis* and maize, it appears that a conservative mechanism exists whereby tasiR-ARFs and miR166 act antagonistically to control the polarity of lateral organs in higher plants [44].

Clusters and super-clusters of 21- and 24-nt phased small RNAs have been identified in the developing inflorescence of rice [37]. The 24-nt type is absent in *Arabidopsis*. Both sizes phased small RNAs are secondary siRNAs, which are triggered by initial cleavages of two 22-nt miRNAs: miR2118 for 21-nt, and miR2275 for 24-nt, respectively, [37, 94]. Following the initial cleavage, OsRDR6 (the rice RNA-dependent RNA polymerase 6 ortholog) converts the single-strand RNAs into double molecules, which are further processed by DCL4 and DCL3b for 21- and 24-nt phased small RNAs, respectively, [93, 94]. It has recently been shown that the 21- and 24-nt phased small RNAs are preferentially or exclusively produced in rice and maize stamens [94], suggesting that they may act at specific stages during male reproductive development.

11 Other Noncoding RNAs

Besides the 20–30-nt small RNAs, there are also large populations of noncoding RNAs with longer lengths, including tRNAs, rRNAs, snRNAs, antisense RNAs, and lncRNAs, etc.

tRNAs and rRNAs have long been known as housekeeping RNAs to function in protein synthesis process. However, this concept has been updated recently as studies in animals and fungi have shown that both tRNAs and rRNAs are capable of producing small RNAs to regulate gene expression or translation [23, 52]. In addition, a potential role of initiator tRNAs in regulating pre-mRNA splicing has also been revealed [40]. The current rice genome annotation has identified over 700 tRNA genes [87], however the regulatory roles of tRNAs, or tRNA-derived small RNAs have yet to be reported in rice.

The well-known snRNA class of small RNAs are associated with spliceosomes and are named U1–U7 snRNAs due to the enrichment of uridines (U) in their sequences [109]. SnRNAs are usually 100–250 nt long [84]. SnRNAs have been shown to associate with ribonucleoproteins to form spliceosomes which regulate pre-mRNA splicing and their sequences are highly conserved across species [39]. One specific class of snRNAs is the small nucleolar RNA (snoRNA) class whose primary function is to guide the modification of rRNAs, as well as some tRNAs and snRNAs [6]. Nascently transcribed pre-rRNA needs to undergo a series of modifications to become mature rRNA. These modifications are mainly carried out by snoRNAs. There are two major families of snoRNAs. The C/D box snoRNAs containing the C box and D box conserved motifs guide 2′-O-methylation, and the H/ACA box snoRNAs with conserved H and ACA box domains and guide pseudouridylation of rRNAs [46, 73]. In a recent computational survey, a total of 118 C/D and 40 H/ACA box snoRNA families are identified in the rice genome [11] (Table 10.3). Some snoRNA genes are found to be present in clusters in the rice genome, especially in intronic regions [61]. Increasing lines of evidence have shown that snoRNAs also participate in the regulation of multiple biological processes in animals, including pre-mRNA splicing, serving as precursors of miRNAs and causing some neuromuscular diseases [45, 47, 85, 110]. So far, no regulatory functions have been identified for plant snoRNAs.

Natural antisense RNAs are endogenous RNA transcripts with sequence complementary to other mRNAs [83]. According to the relationship of the genomic locations of antisense and their corresponding sense RNAs, antisense RNAs can

Table 10.3 SnoRNA families in the rice genome (modified from [11])

Family	Singleton	Multicopies	Families no.	Major functions
C/D box	38	80 (68 %)	118	2′O-methylation of rRNAs, snRNAs, and tRNAs; rRNA processing
H/ACA box	17	23 (58 %)	40	Pseudouridylation of rRNAs and snRNAs; rRNA processing

be classified as *cis*-encoded and trans-encoded. The former are originated from genes fully or partially overlapping with their sense genes but on the different genomic strands [58]; and trans-encoded antisense transcripts are produced from different genomic locus as compared to the sense transcripts, but with sequence complementary to the sense transcripts, therefore having the potential to interact with the sense transcripts in cytosol. Genome-wide screens in *Arabidopsis* and other eukaryotic species have revealed that over 10 % of all mRNAs have putative antisense partners [30, 58, 82]. An earlier screen in rice using full-length cDNAs discovered 687 potential sense-antisense transcript pairs [82], but this number has to be updated as genome-wide tilling array and transcriptome sequencing have uncovered large numbers of transcripts from intergenic regions [35, 58, 66]. A detailed analysis of the rice *OsDof12* mRNA and its endogenous antisense transcript *OsDof12as* revealed that the sense and antisense transcripts are co-expressed in the examined tissues and have a trend of being reciprocally regulated during development but co-regulated under drought and dark treatment [55]. The expression of natural antisense transcripts can also induce the degradation of sense transcripts via the RNA interference pathway, and antisense transcript-derived siRNAs (nat-siRNAs) have been detected in a few sense-antisense transcript pairs [57]. Other functions of natural antisense transcripts, such as causing transcription repression [16], genomic imprinting [114], and DNA methylation [16] of sense RNAs are awaiting further evidence in rice. A recent study has also shown that rice natural antisense transcripts can serve as miRNA precursors to produce miRNAs [65]. A similar study has not been expanded to other species yet.

Transposable elements comprise at least 35 % of the *O. sativa* ssp. *japonica* genome [87], majority of which are the Ty3/gypsy retrotransposons (Table 10.4). Transcription activity has been detected for many transposons, most of which are silenced by the RNAi pathway to produce siRNAs, and these siRNAs will in turn to silence other transposons of the same family [24, 96]. Some transposons can also serve as precursors of miRNAs [86]. Emerging evidence has shown that transposons, either stand alone or within genes, can regulate the expression of neighboring or host genes, as exemplified by the intronic transposon fragment of the well know flowering regulatory gene *FLOWERING LOCUS C* (*FLC*) in *Arabidopsis* [54, 76].

Table 10.4 Transposons in the rice genome (modified from [87])

Class (34.79 %)	Transposons	Fraction of genome (%)
Class I (19.35 %)	LINES	1.12
	SINES	0.06
	Ty1/copia	3.85
	Ty3/gypsy	10.09
	Other class I	3.43
Class II (12.96 %)	hAT	0.38
	CACTA	2.69
	IS630/Tc1/mariner	2.26
	IS256/mutator	3.64
	IS5/tourist	3.26
	Other class II	0.73
Other classes (1.80 %)	NA	NA

Pseudogenes, homologous to known genes but without the ability to encode functional proteins, have recently been proven to be another source of noncoding RNAs [27, 98]. A total of 1,439 pseudogenes have been identified in the *O. sativa* ssp. *japonica* genome in a recent survey [101], which were characterized by the sequence similarity to know genes and the presence of frame shifts or premature stop codons in their sequences. About 75 % of all rice pseudogenes were generated by gene duplication events and the rest were due to retrotransposition events [101]. Among them, at least 12 % have detected transcripts [101].

Long noncoding RNAs (lncRNAs), commonly referred to as noncoding transcripts with lengths longer than 200-nt, have been shown to be involved in the regulation of many essential biological processes and grasped great attention recently [42, 81]. In *Arabidopsis*, lncRNAs have been shown to play roles in the regulation of flowering by mediating the epigenetic modification of the *FLC* locus [26, 99]. A distinct class of lncRNAs generated by the plant-specific RNA

polymerase, Pol V, has been proven to function in RNA-directed DNA methylation through the involvement of the AGO4 protein [89, 112, 113, 126]. In rice, a transcriptome profiling study using next generation sequencing technology has identified 15,708 novel transcriptional active regions (nTARs) [66]. Among them, 51.7 % have no homolog to public protein data [66], some of which may be functional lncRNAs.

Recently, two groups independently characterized a noncoding RNA which regulates the rice photoperiod- or thermo-sensitive genic male sterility (PGMS or TGMS), respectively, [14, 127]. Both PGMS and TGMS are widely used in the two-line system of hybrid rice production and thus play significant roles in the grain production in China. As one of the most widely used female parents for two-line hybrid rice breeding, the TGMS line-Peiai64S (PA64S) was derived from the first spontaneous PGMS line-Nongken58S (NK58S) [67, 77, 92]. Further characterization of the mechanism for determinacy of pollen fertility in the NK58S or PA64S will shed light on the importance of noncoding RNA in rice [14, 127].

12 Future Perspectives

Although genome-wide miRNA and siRNA targets have been identified in rice, so far, characterization of their biological functions is limited. Disrupting miRNA regulation by expression of nontargeted miRNA or over-expression as well as target mimicry of miRNAs are effective approaches to study rice miRNAs function in future. In addition, isolation of the *OsSPL14*^{ipa1}/*OsSPL14*^{Aikawa1} allele in different rice varieties also provides a clue to identify natural variation such as SNPs at miRNA or target loci. Natural variation may lead to changes of gene expression and eventually affect agronomic traits for increase crop yield or adaption in different rice varieties. The exploration of the basic biology of noncoding RNA is just beginning. Shedding light on the complex roles of noncoding RNAs will be a major challenge for future.

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Disease Resistance

11

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Disease is one of the major factors that influence stable rice production. A large increase in the planted area of single high-yield rice varieties has prompted a corresponding increase in diseases. In addition to being a crop that is closely related to human life, rice is a model monocot plant for genomic function research [1]. Plant immunity, or disease resistance, the more frequently used term in crop production, prevents or reduces plant infection from pathogens. Studies of the molecular basis of rice immunity are therefore of great interest both for gaining mechanistic knowledge about immunity and for advancing crop improvement. Use of natural genetic disease resistance mechanisms is an economical, effective, and environmentally sound choice to prevent disease damage for the development of Green Super Rice varieties [2].

1 Major Diseases of Rice

1.1 Bacterial Diseases

The biotroph *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the causal agent of bacterial blight of rice (*Oryza sativa* L.) and depends, in part, on a type III secretion system for pathogenicity [3]. The bacterium invades the xylem tissue of rice, either through hydathodes or wounds, leading to systemic infection along the xylem vessels [4]. The biotroph *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*), which is closely related to *Xoo*, causes rice bacterial streak disease via stomata or wounds. In contrast to *Xoo*, *Xoc* is limited to the intervascular regions of infected plants, leading to the disease's characteristic leaf streaking [5]. The two bacterial diseases are controlled by quarantine regulations in some countries; for example, bacterial blight is quarantined in the United States and Japan, and bacterial streak is quarantined in China [5, 6].

1.2 Fungal Diseases

The hemibiotroph *Magnaporthe oryzae* causes rice blast disease. It can infect all aboveground parts of a rice plant at multiple growth stages. It also infects rice roots [7], although rice root disease caused by *M. oryzae* has not been reported. This pathogen can also infect barley with artificial inoculation [8]. Sheath blight is another devastating rice fungal disease caused by the soil-borne necrotrophic *Rhizoctonia solani* [9]. This organism is pathogenic to several hundred plant species, both monocots and dicots, and is one of the most common crop diseases. The hemibiotroph *Ustilaginoidea virens* causes false

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smut disease in rice. This disease is visible only after panicle exsertion. False smut reduces both seed quality and grain yield. The fungus produces ustiloxin, which is toxic to humans and domestic animals [10].

1.3 Viral Diseases

Rice grassy stunt virus (RGSV) disease is caused by a single-stranded RNA virus [11]. This disease is transmitted by the brown planthopper and naturally infects only rice. Brown planthopper disperses RGSV by flying from infected fields to newly planted rice fields in distant areas [12]. Rice stripe virus is a member of the genus *Tenuivirus*, which consists of six members, including RGSV. It is transmitted by the small brown planthopper *Laodelphax striatellus* in a persistent manner [13]. Tungro is caused by two viruses: a single-stranded RNA virus, the rice tungro spherical virus (RTSV), and a double-stranded DNA virus, the rice tungro bacilliform virus (RTBV) [14]. Both RTBV and RTSV are transmitted by several *Nephotettix* species and *Recilia dorsalis*. Rice can be infected with RTBV or RTSV alone, or coinfected with both [15]. Rice black streaked dwarf virus is a double-stranded RNA virus [16] that is transmitted in a persistent manner by planthoppers [15].

2 Plant Immunity

2.1 Two Classes of Genes in Plant Immunity

Plant immunity is regulated by a large number of genes that can be divided into two classes [17]. One class comprises the receptor genes that include pattern recognition receptor (*PRR*) genes and gene-for-gene disease resistance (*R*) genes. PRRs are cell surface membrane receptors, and R proteins can be either cell surface or cytoplasmic receptors [18]. However, most R proteins are nucleotide-binding-site-leucine-rich repeat (NBS-LRR) type localized in the cytoplasm. The other class of genes involved in plant immunity is composed of defense-responsive genes, which encode plasma membrane, cytosolic, nuclear, or extracellular proteins that function downstream of PRR or R proteins, either as activators or repressors in defense signaling. Compared with the number of defense-related receptor genes, the number of defense-responsive genes in a given plant genome is large.

2.2 Current Model of Plant Immunity

As in mammals, immunity in plants can be classified as innate immunity and induced or systemic immunity, although the two cannot be mutually excusive. Plants respond to pathogen invasion by using a two-branched innate immune system [18, 19] (Fig. 11.1). On the surface of the host cell, general pathogenic microbial elicitors called pathogen-associated molecular patterns are recognized by host PRRs, which results in pathogen-associated molecular pattern-triggered immunity (PTI) or basal resistance. The known pathogen-associated molecular patterns include bacterial flagellin, lipoprotein, peptidoglycan, lipopolysaccharide, elongation factor, cold shock protein, and sulfated peptide; fungal chitin, xylanase, and ergosterol; and oomycete glucan, transglutaminase, and lipid-transfer protein, which are relatively conserved quite widely across genera or more narrowly within a genus during evolution [20, 21]. Direct interaction between PRRs and pathogen-associated molecular patterns is required for the initiation of PTI. Successful pathogens can overcome PTI by deploying secreted effectors resulting in effector-triggered susceptibility (Fig. 11.1). The known effectors include a medley of toxins and proteins secreted by the bacterial type III secretion system and Arg-X-Leu-Arg (X representing any amino acid) motif-containing proteins secreted by fungi and oomycetes [22–24]. Plants have developed R proteins to perceive race-specific effectors and initiate effector-triggered immunity (ETI; Fig. 11.1) that is also called race-specific resistance or gene-for-gene resistance [25]. R proteins

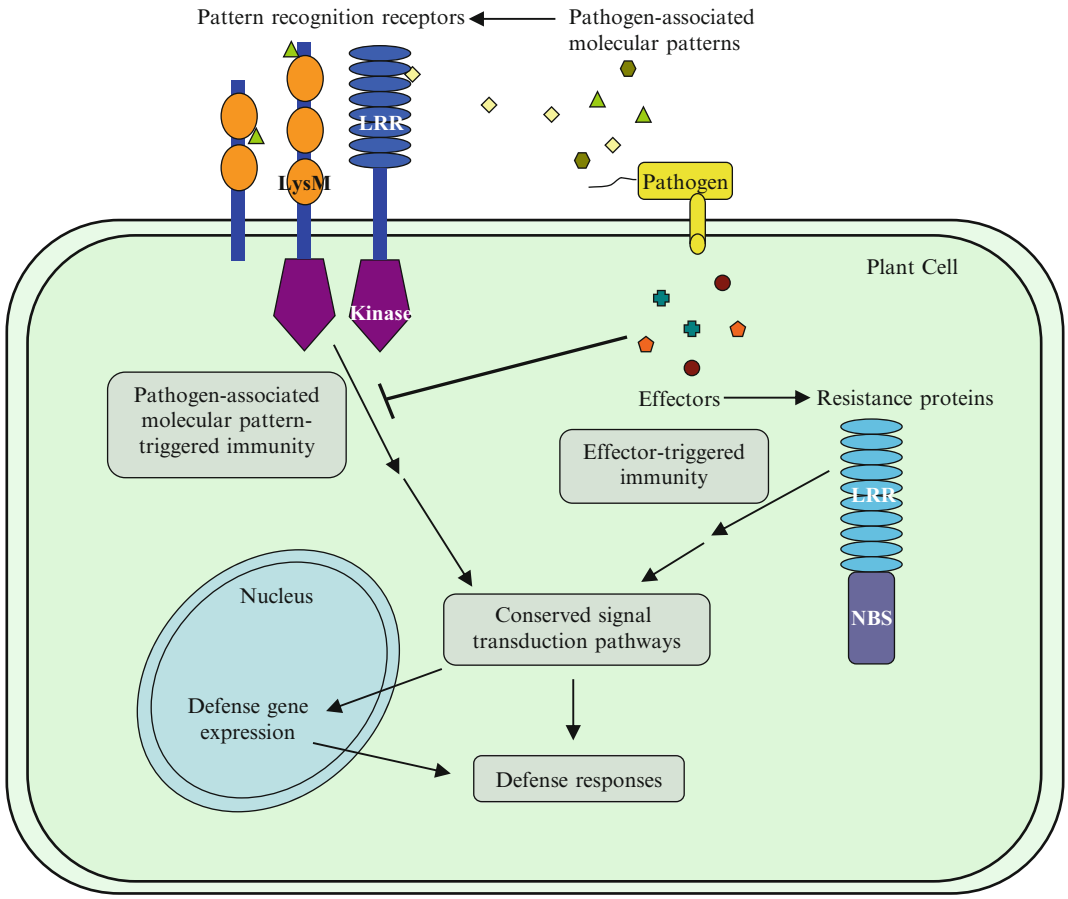

Fig. 11.1 Plant two-branched innate immune system. *LRR* leucine-rich repeat, *LysM* lysine motif, *EGF-like* epidermal growth factor-like, *NBS* nucleotide-binding site

directly or indirectly interact with pathogen effectors to initiate ETI. However, there is a continuum between PTI and ETI, because pathogen-associated molecular patterns and effectors as well as PRRs and R proteins cannot be strictly distinguished [18]. The PRR- and R protein-initiated defense transduction pathways use cross talk or partially overlap [17].

Induced immunity confers enhanced resistance against subsequent attack by a wide array of pathogens. This type of immunity can be divided into two classes: systemic acquired resistance and induced systemic resistance [26]. Some phytohormones, such as salicylic acid, jasmonic acid, and ethylene, have been implicated as signals associated with systemic defense responses [27]. Systemic acquired resistance is associated with systemic accumulation of salicylic acid that can induce a subset of pathogenesis-related (*PR*) genes [28]. Systemic acquired resistance signaling appears to partially overlap with PTI and ETI [18]. Induced systemic resistance is caused by colonization by certain nonpathogens, such as several *Pseudomonas* and *Bacillus* species, in the root and is rare in nature. Generally, induced systemic resistance requires JA and ethylene. However, Induced systemic resistance (ISR) is neither associated with enhanced biosynthesis of either jasmonic acid or ethylene, nor with massive changes in pathogenesis-related gene expression [29].

2.3 Qualitative Versus Quantitative Resistance

According to the speed and strength of plant response to pathogen invasion, plant resistance against pathogen infection is divided into qualitative (or complete, or vertical) resistance conferred by a single or a few genes and quantitative (or partial, or horizontal) resistance mediated by multiple genes or quantitative trait loci (QTLs). Typically, qualitative resistance is considered to be mediated by *R* genes and is pathogen race specific. However, accumulating data suggest that some *PRRs* can also mediate a high level of resistance to pathogens [18]. Furthermore, a defense-responsive gene can also confer a high level of resistance, such as rice *OsDR10* against *Xoo* [30]. Thus, we define qualitative resistance as being conferred by major disease resistance (*MR*) genes that can mediate a rapid and high-level immune response in a plant–pathogen interaction. Quantitative resistance is frequently considered to be broad-spectrum and durable resistance and is the only form of resistance for plants against some types of pathogens [31]. A few genes contributing to resistance QTLs have been identified as components of the defense signaling network downstream of *R* and *PRR* genes [6, 17, 32–34].

3 A Glimpse of Molecular Mechanisms of Rice–Pathogen Interactions

Characterized *MR* genes and genes contributing to resistance QTLs in rice–pathogen interactions are accumulating, which provides a basis for understanding the molecular mechanisms of both qualitative and quantitative resistance.

3.1 Qualitative Resistance

More than 30 and 70 *MR* genes that mediate qualitative race-specific resistance against *Xoo* and *M. oryzae*, respectively, have been named [35–37]. Only a few *MR* genes conferring resistance to different viral disease have been reported [38–41]. No *MR* genes conferring resistance to other major rice diseases, such as bacterial streak, sheath blight, and false smut, have been reported so far [42–44]. Thus, our understanding of rice qualitative resistance is based mostly on the rice–*M. oryzae* and rice–*Xoo* systems.

3.1.1 Qualitative Resistance to *M. oryzae*

Of the more than 70 named rice *MR* genes against *M. oryzae*, 16 of them have been molecularly characterized [45–48]. Among these characterized genes, 15 (*Pia*, *Pib*, *Pita*, *Pi9*, *Pi2*, *Piz-t*, *Pi36*, *Pi37*, *Pikm*, *Pit*, *Pi5*, *Pid3*, *Pikh*, *Pikp*, and *Pik*) encode NBS-LRR-type proteins, the typical R-type protein, and only one *MR* gene (*Pi-d2*) encodes non-NBS-LRR protein. All the *MR* genes confer dominant resistance to *M. oryzae*.

NBS-LRR proteins can recognize pathogen effectors either directly or indirectly through an accessory protein [49]. The MR protein Pita binds directly to the *M. oryzae* effector AvrPita both in vitro and in yeast two-hybrid assays, which is a unique example of direct effector recognition in rice [50]. The special feature in rice–*M. oryzae* interaction is that cooperation of two NBS-LRR genes is required for race-specific qualitative resistance. For example, *Pikm1-TS* and *Pikm2-TS* at the *Pikm* locus of chromosome 11 are both required for *Pikm*-mediated resistance [51]; *Pikp* and *Pik*, the multiple alleles of the *Pikm* locus, also need two adjacent NBS-LRR genes to confer resistance [47, 48]. The same situation has also been identified at the *Pia* locus of chromosome 11 and the *Pi5* locus of chromosome 9 [46, 52]. Understanding how these two MR proteins work together will provide new insights into the mechanism of the rice–*M. oryzae* interaction.

The three characterized NBS-LRR-type *MR* genes, *Pi9*, *Pi2*, and *Piz-t*, which confer a relative broad-spectrum resistance against *M. oryzae*, belong to the same tandem clustered family on chromosome 6 [53–55]. *Pi9* originates from the wild rice species *Oryza minuta*. *Pi2* and *Piz-t* are alleles from different local cultivars, and their encoding proteins only have eight amino acid differences [55]. These three genes confer resistance

to different sets of *M. oryzae* strains, but with some overlap. The amino acid differences within the LRR domain may be critical for their resistance specificities [55].

Pi-d2 encodes a plasma membrane-localized receptor-like kinase protein with an extracellular B-lectin domain and an intracellular kinase domain [56]. Pi-d2 belongs to the non-RD subclass of kinases, as do rice MR proteins Xa21 and Xa3/Xa26 for *Xoo* resistance and Arabidopsis PRR FLS2; this similarity implies that Pi-d2 may act as a PRR in innate immunity [57]. The only difference between the resistant *Pi-d2* allele and its susceptibility allele is a single amino acid change in the transmembrane regions of the encoded proteins. This substitution in the transmembrane region does not affect the plasma membrane localization of Pi-d2 [56]. However, how this substitution destroys the resistance function is yet unknown.

3.1.2 Qualitative Resistance to *Xoo*

The molecular mechanisms of rice qualitative resistance to *Xoo* appear largely different from the mechanisms of rice qualitative resistance to *M. oryzae*, although the mechanisms of rice disease resistance remain to be elucidated. First, at least 37 *MR* genes for *Xoo* resistance have been identified, but more than one third of them (*xa5*, *xa8*, *xa9*, *xa13*, *xa15*, *xa19*, *xa20*, *xa24*, *xa25*/*Xa25*(*t*), *xa26*(*t*), *xa28*(*t*), *xa31*(*t*), *xa33*(*t*), and *xa34*(*t*)) confer resistance recessively [35, 37, 58–60]. Second, although only seven *MR* genes (*Xa1*, *Xa3*/*Xa26*, *xa5*, *xa13*, *Xa21*, *xa25*, and *Xa27*) against *Xoo* are molecularly characterized, they encode various types of proteins [37, 61]. The dominant *Xa1* encodes an NBS-LRR protein [62]. The dominant *Xa3*/*Xa26* and *Xa21* encode plasma membrane-localized LRR receptor kinase-type proteins with an extracellular LRR domain, a transmembrane motif, and a cytoplasmic kinase domain [63, 64]. The recessive *xa5* encodes a gamma subunit of transcription factor IIA [65, 66]. The recessive *xa13* and *xa25* encode membrane proteins of the MtN3/saliva family [37, 67]. The dominant *Xa27* encodes an unknown apoplast protein [68]. Thus, rice–*Xoo* is a unique pathosystem to study the interactions between hosts and pathogens.

Resistance Conferred by the *MR* Genes of LRR Receptor Kinase-Type

Xa21 was the first rice *MR* gene characterized [63], and the molecular mechanism of Xa21-mediated resistance has been relatively more intensively studied. This gene confers a race-specific resistance to *Xoo*, although it can mediate a more broad-spectrum resistance than most of the identified *MR* genes against *Xoo* [69]. The Xa21 protein interacts with the Ax21 protein secreted by *Xoo* through its type I secretion system to initiate rice defense signaling [70]. A sulfated 17-amino acid synthetic peptide (AxY^S22) derived from the N-terminal region of Ax21 is sufficient for this initiation. Ax21 is highly conserved in many *Xanthomonas* species and is also present outside the *Xanthomonas* genus, such as plant pathogen *Xylella fastidios* and the opportunistic human pathogen *Stenotrophomonas maltophilia*. Thus Ax21 is considered a pathogen-associated molecular pattern, and Xa21 can be classified both as a PRR and an R protein [70].

Several components functioning downstream of Xa21 in defense signaling have been identified [71]. The XB24/ATPase physically associates with the juxtamembrane motif of Xa21 and enhances Xa21 autophosphorylation that keeps the Xa21 protein in an inactive status [72]. Upon recognition of sulfated Ax21, the Xa21 kinase disassociates from XB24 and is activated, which results in the initiation of downstream defense responses. XB3/E3 ubiquitin ligase, which is transphosphorylated by Xa21 in vitro and required for full activity of Xa21, may serve to activate a downstream mitogen-activated protein kinase (MAPK) cascade [73]. On the other hand, XB15/PP2C phosphatase binds to Xa21 and dephosphorylates Xa21 to negatively regulate the Xa21-mediated innate immune responses [74]. XB10/WRKY62 blocks the activation of defense-related genes and negatively regulates the Xa21-mediated defense response to *Xoo* [75]. BiP3/ER-localized chaperone HSP70, which is involved in processing and degradation of Xa21, interferes with Xa21-mediated immunity [76].

Xa3/Xa26 (also named *Xa3* and *Xa26*) confers race-specific resistance to *Xoo* as well, but with a resistance spectrum different from *Xa21* [64, 77, 78]. The LRR domains of both Xa3/Xa26 and Xa21 are subject to positive selection [79]. Domain swapping analyses suggest that the LRR domains of Xa3/Xa26 and Xa21 are the important determinants of race-specific recognition during rice–*Xoo* interactions; in addition, the juxtamembrane motif of Xa3/Xa26 also appears to contribute to resistance specificity [78]. The kinase domain of Xa3/Xa26 can partially replace the function of the kinase domain of Xa21, or vice versa, in *Xoo* resistance, suggesting that the defense signaling pathways initiated by the two proteins may be partially conserved [78].

The *Xa3/Xa26* orthologs or alleles *Xa3/Xa26-2* from wild rice *O. officinalis* and *Xa3/Xa26-3* from wild rice *O. minuta*, which encode proteins having amino acid differences from the Xa3/Xa26 protein, can mediate a similar spectrum of resistance against *Xoo* [80]. Thus the resistance function of *Xa3/Xa26* locus, which originated at last 7.5 million years ago, appears to have been relatively conserved during evolution [80]. Additionally, rice cultivars carrying *Xa3/Xa26* have been widely used in rice production in China for a long period of time. These results suggest that the *Xa3/Xa26* locus may confer durable resistance. Thus, further study may be worthwhile to determine whether Xa3/Xa26 recognizes a pathogen-associated molecular pattern to initiate defense response.

Another similarity between *Xa3/Xa26* and *Xa21* is that the functions of both genes are dose dependent. The expression of *Xa3/Xa26* and *Xa21* increases gradually during development; in addition, *Xa3/Xa26* has a higher expression level in a *japonica* background than in an *indica* background [78, 81]. This transcriptional feature results in the two genes conferring a higher level of resistance to *Xoo* at the adult stage than at the seedling stage and *Xa3/Xa26* mediating a higher level of resistance in a *japonica* background than in an *indica* background. Given the genetic, structural, and functional characteristics of *Xa3/Xa26* and *Xa21*, further study may also be required to determine whether Xa3/Xa26 is also a PRR in addition to being an R protein.

Several defense-responsive genes functioning in the *Xa3/Xa26*-initiated signal transduction pathway have been identified. *WRKY13* and *WRKY45-2*, encoding WRKY-type transcription factors, both positively regulate rice resistance to *Xoo* and *M. oryzae*, with *WRKY13* putatively functioning upstream of *WRKY45-2* in rice–*Xoo* interaction [82–84]. *OsDR10*, a rice tribe-specific gene, negatively regulates rice resistance to *Xoo*, and it likely functions upstream of *WRKY13* in an *Xa3/Xa26*-initiated defense pathway [30]. *C3H12*, encoding a CCCH-type zinc finger nucleic acid-binding protein, positively regulates rice resistance to *Xoo* via functioning upstream of *WRKY45-2* [34]. *OsDR8* encodes a protein involved in thiamine biosynthesis and positively regulates rice resistance against *Xoo* and *M. oryzae*; this gene also functions in *Xa3/Xa26-initiated* defense pathway in rice–*Xoo* interaction [85].

Resistance Conferred by the *MR* Genes of MtN3/Saliva-Type

The fully recessive *R* gene *xa13*, which mediates race-specific resistance to Philippine *Xoo* strain PXO99, encodes a plasma membrane protein [67, 86]. Promoter swapping analysis confirms that the dominant allele of recessive *xa13*, *Xa13* (also named *Os8N3* and OsSWEET11), is a susceptibility gene that is specific to a *Xoo* strain carrying *pthXo1*, which encodes the transcription activator-like (TAL) effector PthXo1 [87–89]. PthXo1 transcriptionally activates the dominant *Xa13* gene by directly binding to a *cis*-acting element, the UPT_{PthXo1} box, in the *Xa13* promoter [87, 90, 91]. The *Xoo* strain PXO99 that secretes PthXo1 cannot induce recessive *xa13* due to the mutation of the UPT_{PthXo1} box in the *xa13* promoter [67]. Thus, the recessive *xa13* is a mutant of the *Xoo* susceptibility gene *Xa13*. It stands out from other characterized dominant *MR* genes in that its functions in disease resistance are due only to the loss of *Xoo*-induced transcriptional motivation caused by natural selection.

Both dominant *Xa13* and recessive *xa13* are required for reproductive development [67]. However, *Xoo* strain PXO99 uses dominant *Xa13* to facilitate its invasion of rice. Copper, an essential

micronutrient of plants, is also an important element for a number of pesticides in agriculture. Copper can inhibit *Xoo* growth, and PXO99 is more sensitive to copper than other *Xoo* strains. The Xa13 protein interacts with two copper transporter-type proteins, COPT1 and COPT5, to promote removal of copper from xylem vessels, where *Xoo* multiplies and spreads to cause disease [86]. PXO99 cannot induce recessive *xa13*. The copper levels in rice plants carrying the recessive *xa13* gene can inhibit PXO99 growth and thus plants are resistant to PXO99.

The recessive *xa25* gene, which is also named *Xa25*(*t*), is evolutionarily closely related to *xa13*/*Xa13* and confers race-specific resistance to Philippine *Xoo* strain PXO339 [37, 92]. The expression of dominant *Xa25* but not recessive *xa25* is specifically induced by PXO339. The *xa25*-mediated resistance is influenced by the developmental stage. In transgenic plants with the genetic background of dominant *Xa25*, the transgene *xa25* regulated by its native promoter functions as a recessive gene. However, rice plants which were heterozygous at the *xa25* locus were susceptible to PXO339 at the seedling stage, but became resistant to PXO339 at the adult stage. The dominance reversal characteristic of *xa25* appears to be associated with suppression of PXO339-induced activation of dominant *Xa25* at the adult stage [37].

Resistance Conferred by the *MR* Genes of Other Types

Xa1 is the only NBS-LRR-type *MR* gene identified so far for *Xoo* resistance [62]. It confers a high level of race-specific resistance to race 1 strains of *Xoo* in Japan, the most dominant race in Japan. *Xa1* expression is induced by wounding and pathogen infection.

The recessive *xa5* mutant is an allele of *Xa5* encoding the gamma subunit of transcription factor IIA (TFIIAγ) [65]. TFIIAγ is involved in the recruitment of the basal transcription machinery by eukaryotic transcription factors. The *xa5* allele contains a missense mutation that does not seem to influence its function in the recruitment of the basal transcription machinery [66]. The virulent transcriptional activity of *Xoo* TAL effector Avrxa5 on rice gene(s) may require TFIIAγ; the *xa5* mutation is an adaptation to evade the virulence of the effector [93]. However, how the *xa5* mutation is involved in interfering with TAL effector function is yet unknown.

Xa27 functions differently from its susceptibility allele by differential expression during rice–*Xoo* interaction. The resistance and susceptibility alleles of *Xa27* encode identical proteins, but the two alleles have nucleotide differences in their promoters [94]. The promoter differences result in the *Xa27* promoter, but not the promoter of the susceptibility allele, harboring a *cis*-acting element $UPT_{AvrXa27}$ box [95]. No expression was detected in the susceptible plants or in *Xa27-carrying* plants challenged with compatible *Xoo* strains. Expression of the resistance allele induced by *Xoo* strains delivering a TAL effector AvrXa27 is involved in *Xa27*-mediated resistance [94]. The AvrXa27 effector directly binds to the $UPT_{AvrXa27}$ box to induce *Xa27* [95]. AvrXa27 is dependent on Xa5, the TFIIAγ, to activate *Xa27* transcription that triggers resistance to *Xoo*, whereas the recessive *xa5* attenuates *Xa27-mediated* resistance [96]. In addition, *xa5-mediated* resistance is also down-regulated in *xa5* and *Xa27* double homozygote plants. However, the mechanism of down-regulation of *xa5-mediated* resistance is unknown [96].

3.1.3 Qualitative Resistance to Viruses

Currently at least nine dominant *MR* genes conferring resistance to viruses, which have been identified from other crops (e.g., potato, tomato, tobacco, soybean, and bean) and model species (*Arabidopsis thaliana*), all fall into the NBS-LRR class of resistance genes, aside from two *Arabidopsis* genes, *RTM1* and *RTM2* [97]. About half of the ~200 known virus resistance genes in plants are recessively inherited [98]. In contrast to the dominant resistance genes, the recessive genes for viral disease resistance are more likely to be constitutive components of cellular activity. The natural mutations of components of the eukaryotic translation initiation complex are usually involved in recessive resistance to specific RNA viruses [98, 99]. This recessive resistance is quantitative resistance

in many cases, but also it can be qualitative [44]. Rice cultivars with a dominant *MR* gene to RGSV introduced from a wild rice species, *O. nivara*, have been widely used in rice production [38, 39], but it has not been characterized molecularly. A recessive *MR* gene mapped within an approximately 200-kb region of chromosome 7 has been reported as associated with resistance to RTSV. The eukaryotic translation initiation factor 4G (eIF4G) gene is a candidate for RTSV resistance [41]. *Rymv1* encodes the isoform of eIF4G and is a recessive *MR* gene of rice against rice yellow mottle virus [40].

3.2 Quantitative Resistance

No *MR* genes against rice sheath blight, false smut, bacterial streak, or many viral diseases have been reported so far. Thus, quantitative resistance is presently the only form of resistance that can be used for rice improvement against these diseases. A large number of resistance QTLs against different rice diseases including blast, sheath blight, false smut, bacterial blight, bacterial streak, and some viral diseases have been identified [6]. Most of these resistance QTLs are minor ones that each only explains less than 10 % phenotypic variation and others are major QTLs that each explains more than 10 % phenotypic variation.

Various types of genes have been reported to contribute to quantitative resistance, although the association of some genes with resistance QTL remains to be established [6, 100]. Defense-responsive genes are an important contributor to quantitative resistance. Eleven genes (*NRR*, *WRKY13*, *WRKY45*, *GH3-1*, *GH3-2*, *GH3-8*, *pi21*, *OsDR8*, *MPK6*, *Pb1*, and *C3H12*) contributing to resistance QTLs have been identified, at least ten of which are defense-responsive genes. In addition, some receptor-like genes in rice–pathogen interactions also contribute to quantitative resistance. Furthermore, multiple genes can contribute to a single resistance QTL, most likely because of tight linkage. Studies of the molecular mechanisms of these genes in rice–pathogen interactions provide a glimpse of the characteristics of rice quantitative resistance. First, resistance QTLs can be the components of either the *MR* gene-initiated defense pathway or the basal defense pathway. Second, resistance QTLs can function either as a positive regulator or a negative regulator in defense responses. Lastly, a single minor resistance QTL can mediate broad-spectrum resistance.

3.2.1 Genes Contributing to Major Resistance QTL

pi21 and *Pb1*, which act against leaf blast and panicle blast caused by *M. oryzae*, respectively, are two major resistance QTLs. *pi21* is a recessive gene conferring durable resistance, which encodes a loss-of-function mutation in a cytoplasmic proline-rich protein [101]. *Pb1* encodes an atypical NBS-LRR protein and confers adult resistance [102].

WRKY45, which encodes a WRKY-type transcription factor, contributes to a major resistance QTL against *Xoo* [100]. There are at least two alleles, *WRKY45-1* and *WRKY45-2*, at the *WRKY45* locus [84]. *WRKY45-1* negatively regulates rice resistance to *Xoo* and *Xoc*, whereas *WRKY45-2* positively regulates rice resistance to *Xoo* and *Xoc*. However, both alleles are positive regulators of rice resistance against *M. oryzae*. The opposite roles of the two allelic genes in rice–bacteria interactions appear to be due to their mediation of different defense signaling pathways. *WRKY45-2* functions in the *MR* gene *rbr2*-initated pathway in rice–*M. oryzae* interaction, in addition to playing a role in the *Xa3*/*Xa26-initiated* defense pathway in rice–*Xoo* interactions [84, 103].

Several *OsGLP* gene family members, encoding germin-like proteins, also referred to as oxalate oxidase-like proteins, are clustered in tandem array on chromosome 8 that were found to be associated with a major resistance QTL for resistance to two fungal pathogens, *M. oryzae* and *R. solani* [104]. Using a different mapping population, a minor QTL against *M. oryzae* was associated with the *OsGLP* family members [100]. Suppression of several *OsGLP* members resulted in increased susceptibility of transgenic plants to *M. oryzae* [104].

3.2.2 Genes Contributing to Minor Resistance QTLs

Several genes contributing to minor resistance QTLs also function in *MR* gene-initiated defense pathways. *NRR* contributes to a minor resistance QTL against *Xoo* [100]. It encodes a biochemically unknown protein, which interacts with NH1, a key regulator controlling the onset of systemic acquired resistance. NRR negatively regulates *Xa21*-mediated race-specific resistance and basal resistance to *Xoo* [105]. *WRKY13*, functioning in the *Xa3*/*Xa26*-initiated defense pathway to *Xoo* as described above, contributes to a minor resistance QTL against both *Xoo* and *M. oryzae* [32, 33, 82, 83]. *OsDR8* encoding a thiamine synthesis-related protein also contributes to a minor QTL against both *Xoo* and *M. oryzae* [32, 85]. Transgenic plants with suppressed *OsDR8* show reduced resistance to *Xoo* and *M. oryzae* and a lower level of thiamine [85]. Exogenous application of thiamine complemented the compromised defense of the *OsDR8-suppressing* plants, suggesting that accumulation of thiamine regulated by *OsDR8* may be essential for rice resistance to *Xoo* and *M. oryzae* [85]. *OsDR8* also functions in the *MR* gene *rbr2-initated* pathway in rice–*M. oryzae* interaction, in addition to functioning downstream of *Xa3*/*Xa26* in a rice–*Xoo* interaction [84, 103]. *C3H12*, functioning downstream of *Xa3*/*Xa26*-initiated defense signaling as already described, contributes to a minor QTL against *Xoo* [34].

GH3-1, *GH3-2*, and *GH3-8*, which belong to the *GH3* gene family, function in basal resistance by suppressing auxin signaling. The three genes correspond to different minor resistance QTLs against *M. oryzae* and *Xoo* [32, 33, 100]. GH3-2 and GH3-8 positively regulate rice resistance by preventing auxin-induced cell-wall loosening [33, 106]. GH3-1 appears to have a biochemical function similar to GH3-2 and GH3-8 and positively regulates resistance to *M. oryzae* [107]. MAPK cascades play important roles in basal resistance [108, 109]. A MAPK gene, *MPK6*, is associated with a minor resistance QTL for resistance to *M. oryzae* [32]. The functions of MPK6 in rice–pathogen interactions will be discussed in the following section.

3.3 Systemic Acquired Resistance

Arabidopsis NPR1 is a key regulator of salicylic acid-mediated systemic acquired resistance. NPR1 is believed to regulate *PR1* gene expression through interaction with TGA transcription factors [110]. Transgenic rice plants overexpressing *NH1*, a rice homologue of *NPR1*, have a high level of resistance to *Xoo*. NH1 interacts with rTGA2.2, a rice bZIP transcription factor similar to Arabidopsis TGA2, suggesting that the rice NH1-rTGA2.2 interaction is similar to that of the NPR1-TGA2 interaction in Arabidopsis. However, *NH1*-overexpressing rice plants constitutively express defense genes, contrasting to findings from Arabidopsis overexpressing NPR1, in which defense gene expression was not observed until induction by pathogen infection. Rice *NH1* has revealed similarities as well as differences between rice and Arabidopsis with regard to defense responses [111].

MPK6 is a two-faced player in the rice–*Xoo* interactions; it functions both as an activator and a repressor in rice resistance to *Xoo* [112, 113]. It negatively regulates systemic acquired resistance in the rice–*Xoo* interaction; *MPK6*-suppression/knockout plants showed enhanced resistance to *Xoc* and increased expression of *PR1a*, the marker gene of systemic acquired resistance, in systemic tissues after local infection of *Xoo*. In contrast, MPK6 positively regulates rice local resistance to *Xoo*. Nuclear localization of MPK6 is essential for local resistance, suggesting that modulating the expression of defense-responsive genes through transcription regulators may be the primary mechanism of MPK6-mediated local resistance. MPK6 also functions as a positive regulator in rice resistance to *Xoc* and *M. oryzae* [31, 113]. Thus, MPK6 has multifaceted roles in rice–pathogen interactions.

3.4 Phytohormone Signaling in Rice–Pathogen Interactions

Increasingly, plant hormones are found to be involved in plant–pathogen interactions, in addition to the well-known defense-related hormones, ethylene, jasmonic acid, and salicylic acid [114, 115].

The phytohormone signaling pathways in rice defense responses or disease development are mostly studied using the rice–*Xoo* and rice–*M. oryzae* systems.

Accumulating data suggest that jasmonic acid and salicylic acid play roles in *rice–Xoo* interactions. Resistance against biotrophic and hemibiotrophic pathogens is usually regulated by the salicylic acid-dependent pathway, whereas resistance against necrotrophic pathogens is usually controlled by the jasmonic acid/ethylene-dependent pathway [116]. Salicylic acid- and jasmonic acid/ethylene-dependent defense signals can interact with each other either synergistically or antagonistically [28]. *Xoo* is a biotrophic pathogen [5]. Rice *WRKY13*-mediated resistance against *Xoo* has been reported to be accompanied by increased accumulation of salicylic acid and a reduced level of jasmonic acid [82, 117]. Suppression of defense-responsive genes *MPK6* or *OsDR10* are also associated with increased salicylic acid and reduced jasmonic acid levels [30, 112, 113]. *WRKY45-1* negatively regulates *Xoo* resistance, which is associated with increased accumulation of salicylic acid and jasmonic acid, and its allele, *WRKY45-2*, positively regulates *Xoo* resistance, which is associated with increased accumulation of jasmonic acid, but not salicylic acid [84]. *C3H12*, which functions upstream of *WRKY45-2* in the defense signal transduction pathway against *Xoo* positively regulates *Xoo* resistance, which is also associated with increased accumulation of jasmonic acid, but not salicylic acid [34]. However, *GH3-2* and *GH3-8* positively regulate basal resistance to *Xoo*, which is accompanied by reduced accumulation of both salicylic acid and jasmonic acid [33, 106]. These results suggest that multiple mechanisms may be involved in rice resistance against *Xoo*, and these may include antagonistic or synergistic cross talk of salicylic acid- and jasmonic acid-dependent signaling, salicylic acid and jasmonic acid-independent signaling, and jasmonic acid-dependent signaling. This hypothesis is supported by the observation that jasmonic acid- and salicylic acid-dependent pathways appear to have crisscross roles in a major disease resistance gene *Xa3*/*Xa26*-mediated resistance [118].

M. oryzae is a hemibiotrophic pathogen. Rice plants overexpressing *AOS2*, which encodes an allene oxide synthase involved in jasmonic acid synthesis, showed enhanced resistance to *M. oryzae* and an increased level of jasmonic acid [119]. Activation of *WRKY13*, *WRKY45-1*, or *WRKY45-2* can promote rice resistance to *M. oryzae* [82, 84]. As already discussed, manipulating the expression of *WRKY13*, *WRKY45-1*, or *WRKY45-2* results in the activation or suppression of jasmonic acid, salicylic acid, or both hormone signaling pathways in rice–*Xoo* interactions. Thus, it would be worthwhile to elucidate whether different pathways associated with jasmonic acid and salicylic acid are involved in rice resistance to *M. oryzae* in further studies.

Increased ethylene biosynthesis is required for rice resistance to *M. oryzae* [120, 121]. However, this hormone is a negative signaling molecule in rice resistance to *Xoo*. Rice OsEDR1 is a sequence ortholog of Arabidopsis EDR1. OsEDR1 transcriptionally promotes ethylene synthesis that, in turn, suppresses jasmonic acid- and salicylic acid-associated defense signaling and results in rice susceptibility to *Xoo* [121].

The plant growth hormone auxin plays a complicated role in plant–pathogen interactions. It promotes either pathogenesis or plant defense [122]. Indole-3-acetic acid is the major form of auxin in most plants, including rice. *Xoo*, *Xoc*, and *M. oryzae* are able to synthesize Indole-3-acetic acid [33]. Disease development caused by the three types of pathogens in rice is associated with accumulation of Indole-3-acetic acid [33, 106]. Indole-3-acetic acid induces the expression of expansins, the cell-wall loosening proteins, and results in rice plants being vulnerable to pathogen infection. Rice *GH3-2* and *GH3-8*, encoding Indole-3-acetic acid-amido synthetases that inactivate Indole-3-acetic acid by catalyzing the formation of Indole-3-acetic acid–amino acid conjugate, enhance rice basal resistance to *Xoo*, *Xoc*, and *M. oryzae* [33, 106].

Abscisic acid has complex roles in plant–pathogen interactions; it may function as a negative or a positive signal molecule in plant immunity [116, 123]. This hormone functions negatively in rice resistance to *M. oryzae*. Exogenous application

of abscisic acid compromises rice resistance to this pathogen by suppressing the salicylic acid signaling pathway [124, 125]. The role of abscisic acid in the interaction of rice and the bacterial pathogen *Xoo* is not clear. However, *Xoo* induces susceptible rice plants to accumulate more abscisic acid than resistant plants [118]. Thus, further study may target the putative negative role of abscisic acid in the rice–*Xoo* interaction.

Gibberellin has a pathogenic role in rice–*Xoo* and rice–*M. oryzae* interactions. The elongated uppermost internode (*Eui*) gene, encoding a P450 monooxygenase that deactivates biologically active gibberellin, acts as a positive modulator in rice disease resistance. *Eui* knockout mutants in rice show decreased disease resistance to both *Xoo* and *M. oryzae*, while overexpression of *Eui* increases resistance [126]. Viral proteins have also been shown to affect gibberellin signaling components in rice. The outer capsid protein P2 of rice dwarf virus (RDV) interacts with rice *ent-kaurene* oxidase and affects the production of gibberellin. The expression of *ent*-kaurene oxidase is reduced in the infected plant. The level of endogenous gibberellin 1 in the RDV-infected plants is lower than that in healthy plants. Exogenous application of gibberellins 3 to RDV-infected rice plants restores the normal growth phenotypes. These observations indicate that RDV modulates gibberellin metabolism to promote disease symptoms in rice [127].

4 Perspective

To meet the criteria for development of Green Super Rice, breeding rice with the quality of broad-spectrum and durable disease resistance using genetically defined resources is one of the principal goals of rice improvement [2, 128]. Race-specific qualitative disease resistance is rarely subject to environmental impact, but is easily broken down by rapid evolution of the pathogen. Quantitative resistance is more broad spectrum and durable [17]. It is the most important or only form of resistance to necrotrophic pathogens and even some biotrophic pathogens (e.g., *Xoc*). For major resistance QTL, marker-assisted selection would be an efficient approach for using these resources. Minor resistance QTLs can be used for the improvement of rice broad-spectrum and even durable resistance by manipulating their expression [32, 100]. In addition, approaches directed at harnessing PRR-mediated immunity will be a useful strategy for enhancing resistance in agricultural crops. For example, rice varieties carrying *Xa21* have robust resistance to diverse strains of *Xoo* [129]. Generation and expression of chimeric host *PRR* genes and *PRR* genes from other species are also a viable strategy for engineering resistance [130]. We anticipate that more genes for improvement of rice disease resistance will be available.

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Insect Resistance

12

Guangcun He, Bo Du, and Rongzhi Chen

1 Introduction

The cultivated rice *Oryza sativa* was domesticated about 10,000 years ago along the river valleys of South and Southeast China and Asia [14]. Earliest recordings of rice are found in Chinese writings of about 3000 BC. In ancient China, rice was described as the most important food plant in the country. In the Book of Odes, which comprises 305 poems and songs ranged from the tenth to the seventh centuries BC, there is a record seeming to describe grasshoppers and stem borer damaging rice. In that time, insect pests were removed manually by hand or by using simple tools or through cultural practices. Tobacco was also used for control of insect pests [127].

Rice grows under a wide range of environmental conditions, but most rice is cultured in irrigated and rainfed lowland rice fields. The warm and humid environment in rice field is suitable for the proliferation of insects. In 1960s, the semidwarf varieties, which are more tolerant and responsive to chemical fertilizer thus more productive, were released. The monoculture of a few prevailing varieties and their derivatives has led to the loss of genetic diversity of rice varieties. Susceptible varieties and heavy fertilizers favor the buildup of pest populations.

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There are hundreds of insect species that have been recorded to feed on rice [29], about 30 species can cause yield loss. Insect pests attack the rice plant from seedling to mature plant on all parts of the plant. The occurrence of insect pests varies depending on the location, time of year, crop varieties and cultural practices. Rice variety without resistance is vulnerable. Insect pests that can cause significant rice yield losses are stem borers, plant hoppers and leafhoppers, leaf folders, and gall midges. The sucking insects, plant hoppers and leafhoppers, transmit viruses as well and cause indirect damage to rice. Insects reduce yields substantially. Average losses due to insects calculated from extensive insecticide trials were 28 % as a worldwide average with 34 % occurring in Asia [13]. In a study in the Philippines, over a 13-year period, yield losses were estimated to be 12.7 % in irrigated rice, ranged from 5 to 71 % in upland rice, and from 2 to 88 % in rainfed wetland rice [70–72]. In recent years, yield loss of 10 % was recorded in China, India, Indonesia, the Philippines, Thailand, and Vietnam due to brown planthopper (BPH) damage. The rise of BPH outbreak might be one of the reasons of rising market price of rice since 2003 [88]. Any decrease in pest damage through effective control means a corresponding increase in rice production.

Reduction in insect damage on rice crop can be achieved by growing resistant varieties, which is the major tactic in the integrated pest management (IPM) of pest [114]. Genetic resistance of a plant to insect is the heritable qualities that enable

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plant to reduce the degree of insect damage it suffers [90]. Rice varieties show different level of resistance to insect from extreme resistance to extreme susceptibility [102]. Variety resistance does not add extra expenditure to the farmer and is easy to be popularized. The adverse effects of resistant rice to insect pests are evident. The physiology and behavior of insects are altered on resistant plants, and the susceptibility of insects to insecticides is increased [57]. The survival and migration potential decreased evidently for BPH on resistant variety [89]. Resistant cultivars are well compatible with biological control agents. Resistance to multiple insects is the objective of rice breeding, which will greatly reduce the consumption of pesticides and decrease pest damage in rice crop [143].

2 Rice Germplasm for Insect Resistance

Rice germplasm collections include wild *Oryza* species and two cultivated species, *O. sativa* and *O. glaberrima*. A large number of rice germplasm have been collected over the world. In China, a total of 64,186 rice accessions were stored in the national genebank at the end of 1995. At the International Rice Research Institute (IRRI), there are over 84,200 accessions in the collection. These germplasm collections provide a rich source of insect resistance genes.

Screening for insect resistance in rice germplasm has been conducted throughout the world, and efficient screening methods have been developed. The screening can be done in the greenhouse or in the field, at the vegetative growth stage or reproductive growth stage. The method employed should give distinctly different reactions for the susceptible and resistant entries. Greenhouse screening at seedling stage is the rapid method for evaluating a large number of germplasm and breeding lines, and the resistant one is further evaluated in the field. IRRI has developed a standard system for evaluation insect resistance in rice which is described in detail in Heinrichs et al. [32]. In IRRI system, the entries are given a score of 0–9 based on degree of plant damage. Grade 0 indicates no damage or immune, and grade 1 indicates highly resistant. Grade 9 indicates severe damage or highly susceptible. In field test, susceptibility ratings of 7–9 mean severe economic losses. For example, in testing rice lines resistance to BPH at seedling stage, when the susceptible TN1 seedlings had died (scored as 9), each seedling was given a score of 0, 1, 3, 5, 7, or 9 (Table 12.1). The resistance score of each variety was inferred from the weighted average of the scores for the seedlings in the line [44]. The system is convenient for germplasm screening and breeding material selection. However, for function characterization of resistance gene, the effect of rice on insects should be taken into account.

Table 12.1 The rating criteria for brown planthopper resistance in rice

Resistance score	Plant state (investigated when Taichung Native 1 plants died)
0	None of the leaves shrank and the plant was healthy
1	1–2 leaves were yellowing or one leaf shrank
3	1–2 leaves were yellowing or one leaf shrank
5	1–2 leaves shrank or one leaf shriveled
7	3–4 leaves shrank or two to four leaves shriveled; the plant was still alive
9	The plant died

Resistance to insect pests has been determined for many accessions in the cultivated varieties and wild species. Because wild rice has survived without help of man, these species have evolved protective mechanisms against major insects. Resistance has been identified in accessions of many wild rice species (Table 12.2). In a screening for BPH resistance germplasm by IRRI, accession resistant to each BPH biotype is about 30 times more frequently in wild rice populations than in cultivated varieties. Furthermore, the resistance to all three biotypes of BPH in wild species was found to be even higher, more than 50 times the occurrence in traditional cultivars [45]. Broad-spectrum resistance is more common in wild species than in the cultivated rice. For resistance to stem borers and leaf folder, it is difficult to find in the cultivated rice, but resistant accessions in several wild species have been

Table 12.2 Insect resistance identified in wild rice germplasm

Resistance to insect	Wild rice species
Brown planthopper *Nilaparvata lugens*	*O. nivara*, *O. rufipogon*, *O. rhizomatis*, *O. eichingeri*, *O. officinalis*, *O. minuta*, *O. australiensis*, *O. latifolia*, *O. ridleyi*, *O. alta*, *O. brachyantha*
Whitebacked planthopper *Sogatella furcifera*	*O. officinalis*, *O. latifolia*, *O. punctata*, *O. eichingeri*, *O. minuta*, *O. ridleyi*
Green leafhopper *Nephotettix virescens*	*O. nivara*, *O. rufipogon*, *O. barthii*, *O. officinalis*, *O. eichingeri*, *O. minuta*, *O. ridleyi*, *O. latifolia*, *O. punctata*
Zigzag leafhopper *Recilia dorsalis*	*O. rufipogon*, *O. latifolia*, *O. officinalis*, *O. perennis*, *O. ridleyi*, *O. minuta*, *O. punctata*
Whorl maggot *Hydrellia philippina*	*O. officinalis*
Gall midge *Orseolia oryzae*	*O. rufipogon*, *O. officinalis*, *O. brachyantha*, *O. ridleyi*, *O. eichingeri*, *O. granulata*
Yellow stem borer *Scirpophaga incertulas*	*O. rufipogon*, *O. alta*, *O. brachyantha*, *O. ridleyi*, *O. minuta*, *O. eichingeri*, *O. punctata*, *O. officinalis*, *O. latifolia*
Striped stem borer *Chilo suppressalis*	*O. rufipogon*, *O. officinalis*, *O. minuta*, *O. punctata*
Leaf folder *Cnaphalocrocis medinalis*	*O. rufipogon*, *O. nivara*, *O. punctata*, *O. brachyantha*, *O. officinalis*

identified (Table 12.2). Such germplasm provides an unprecedented opportunity for rice genetic improvement of insect resistance.

3 Molecular Mapping of Genes for Insect Resistance in Rice

Resistance identified in traditional variety or wild species can be transferred into a modern elite variety for breeding and for gene characterization. Strategy to transfer genes from wild species into cultivars depends on the relatedness of the wild species and the incompatibility barriers. Interspecific hybrids are produced through direct crosses between rice and AA-genome wild species and through hybrid embryo rescue after cross between rice and the wild species with different genome other than AA (i.e., BB, CC, BBCC, CCDD, EE, FF, GG, HHJJ, HHKK) [7, 43]. The alien gene introgression lines are produced through crosses, backcrosses, as well as through embryo rescue. In the process, useful genes like resistance to insects in wild rice are transferred into cultivated rice. Different types of mapping populations are generated for mapping insect resistance genes in rice such as recombinant inbred lines (RILs), doubled haploid (DH), and F_2, F_3, and BC populations. A number of insect resistance genes and QTLs for resistance to insects have been identified and mapped on rice molecular linkage map (Table 12.3). A few QTLs for resistance to rice leaf folder were detected and so far no gene or QTLs for resistance to stem borers are reported.

4 Genes for Brown planthopper Resistance

The BPH, *Nilaparvata lugens*, is one of the most destructive insect pests of rice. BPH sucks phloem sap from rice plant using its stylet mouthparts. Yield loss of 10 % due to BPH damage was recorded in many countries. BPH infestation also causes gassy stunt virus and ragged stunt virus diseases in the field. As BPH has developed resistance to the major insecticides that once were effective for controlling it, growing resistant rice varieties is the first option for controlling the pest. Since the *Bph1* was first identified in cultivated rice [5], a total of 28 genes for BPH resistance have been reported so far. There are 13 BPH resistance genes identified in cultivated rice (*Bph1*-*Bph9*, *Bph17*, *Bph19*(t), *Bph25*, *Bph26*). *Bph5*, *bph7*, and *Bph8* were named based on resistance reaction of the variety to BPH biotypes and inheritance study; their chromosome locations have not been determined. All other 15 BPH resistance genes were identified in rice lines descent from wild rice hybridization programs and have been mapped on chromosomes. These genes from wild species show a broad-spectrum resistance to different biotypes of BPH. Among these genes, *Bph14* and *Bph18* have been cloned via a map-based cloning approach [17, 52], and several others have been fine mapped.

Table 12.3 Insect resistance genes identified in rice

Gene	Germplasm	Chromosome	Linked markers	References
Brown planthopper				
Bph1	Mudgo	12	XNpb248; pBPH4 and pBPH14	[5, 9, 36]
bph2	ASD7	12	KAM3 and KAM5; RM7102 and RM463	[5, 80, 118]
Bph3	Rathu Heenati	6	RM589 and RM588	[49, 50, 59]
bph4	Babawee	6	RM589 and RM586	[50, 59]
bph5	ARC 10550	–		[53]
Bph6	Swarnalata	4	Y19 and Y9	[93]
bph7	T12			[53]
bph8	Chin Saba			[87]
Bph9	Balamawee	12	RM463 and RM5341	[81, 87, 116]
Bph10	*O. australiensis*	12	RG457	[47]
bph11	*O. officinalis*	3	G1318	[35]
Bph12	B14	4	RM16459 and RM1305	[94, 136]
Bph13	*O. eichingeri*	2	RM240 and RM250	[74]
Bph13	*O. officinalis*	3	AJ09230b	[100]
Bph14	B5	3	G1318 and SM1	[17, 110, 126]
Bph15	B5	4	RG1 and RG2	[44, 137]
Bph17	Rathu Heenati	4	RM8213 and RM5953	[117]
Bph18(*T*)	IR65482-7-216-1-2 (*O. australiensis*)	12	RM6869 and R10289S	[51]
Bph19(*T*)	AS20-1	3	RM6308 and RM3134	[12]
bph18(*t*)	*O. rufipogon*	4	RM6506 and RM273	[65, 67]
bph19(*t*)	*O. rufipogon*	12	RM17	[65, 67]
Bph20(*T*)	IR71033-121-15′ (*O. minuta*)	4	B42-B44	[95]
Bph21(*T*)	IR71033-121-15 (*O. minuta*)	12	S12094A-B122	[95]
Bph22(*T*)	*O. glaberrima*			[96]
Bph23(*T*)	*O. minuta*			
bph22(*t*)	*O. rufipogon*	4	RM8212 and RM261	[37]
bph23(*t*)	*O. rufipogon*	8	RM2655 and RM3572	[37]
bph24(*t*)	IR 73678-6-9-B (*O. rufipogon*)			[15]
Bph25(*T*)	ADR52	6	S00310	[82, 83, 138]
Bph26(*T*)	ADR52	12	RM5479	[82, 83, 138]
Gall midge				
GM1	W1263	9	RM219 and RM444	[6, 33]
GM2	Phalguna	4	RM241 and RM317	[77, 85, 121]
gm3	RP2068-18-3-5			[63, 103]
GM4	Abhaya, PTB10	8	RM210 and RM256, RM22550 and RM547	[21, 78, 86]
GM5	ARC5984	12	RM101 and RM309	[21]
GM6	Duokang #1	4	PSM112 and PSM114	[42, 58]
GM7	RP2333-156-8	4	SA598	[106]
GM8	Jhitipiti	8	AR257 and AP19587	[48]
GM9	Line 9			
GM10	BG 380-2			[62]
GM11	CR57-MR1523	12	RM28574 and RM28706	[34]
Small brown planthopper				
*Qsbph2*b	Mudgo	2	RM5791–RM29	[18]
Qsbph3d	Mudgo	3	RM3199–RM5442	[18]

(continued)

Table 12.3 (continued)

Gene	Germplasm	Chromosome	Linked markers	References
*Qsbph1*2	Mudgo	12	I12-17–RM3331	[18]
Whitebacked planthopper				
Wbph1	N22			[112]
Wbph2	ARC10239	6	RZ667	[75]
Wbph3	ADR52	–		[60]
wbph4	Podiwi A8	–		[60]
Wbph5	Diang Marie	–		[60]
Wbph6	Guiyigu	11	RM167	[69]
Wbph7	*O. officinalis*	3	R1925 and G1318	[124]
Wbph8	*O. officinalis*	4	R288 and S11182	[124]
Ovc	Asominori	6	R1954	[135]
qOVA-1-3	Asominori	1	XNpb346 and C112	[134]
qOVA-4	Asominori	4	R1854	[134]
qOVA-5-1	Asominori	5	XNpb251 and R3313	[134]
qOVA-5-2	Asominori	5	C1268	[134]
	IR64	7	RG511-RG477	[26]
qWPH2	*O. rufipogon*	2	RM1285-RM555	[11]
qWPH5	*O. rufipogon*	5	RM3870-RZ70	[11]
Qwph9	*O. rufipogon*	9	RG451-RM245	[11]
Green leafhopper				
GLH1	Pankhari 203	5		[5]
GLH2	ASD7	11		[5]
GLH3	IR8	6		[5]
glh4	Ptb8	3		[113]
GLH5	ASD8, *O. rufipogon*	8		[113]
GLH6	TAPL796	5		[101]
GLH7	Maddani Karuppan			[101]
glh8	DV85			[27]
GLH9	IR28			[2]
glh10	IR36			[3, 4]
GLH11	IR20965-11-3-3			[3, 4]
GLH12	Hashikalmi, Ghaiya, RC10313			[3, 4]
GLH13	Asmaita			[3, 4]
GLH14	ARC11554	4		[109]
Green rice leafhopper				
GRH1	Pe-bi-hun, IR24	5	R569 and C309	[54, 122, 139]
GRH2	Lepedumai	11	C50 and R2458	[25, 54, 140]
GRH3	Rantaj-emas 2	6	C288B, C133A and C81	[104, 105]
GRH4	DV85	3		[25, 54, 140]
GRH5	*O. rufipogon* (W1962)	8	RM3754 and RM3761	[23]
GRH6	SML17, IRGC105715	4	RM5414 and C60248	[22, 123]
qGRH4	*O. rufipogon*	4	RM6997 and RM7051	[23]
qGRH9	*O. glaberrima* (IRGC104038)	9	RM215 and RM2482	[24]
Rice leaf folder				
qRLF-1	Chunjiang 06	1	RM3412 and RM6716	[99]
qRLF-2	Chunjiang 06	2	RM207 and RM48	[99]
qRLF-3	Chunjiang 06	3	RM1022 and RM7	[99]
qRLF-4	Chunjiang 06	4	RM3276 and RM255	[99]
qRLF-8	Chunjiang 06	8	RM72 and RM331	[99]

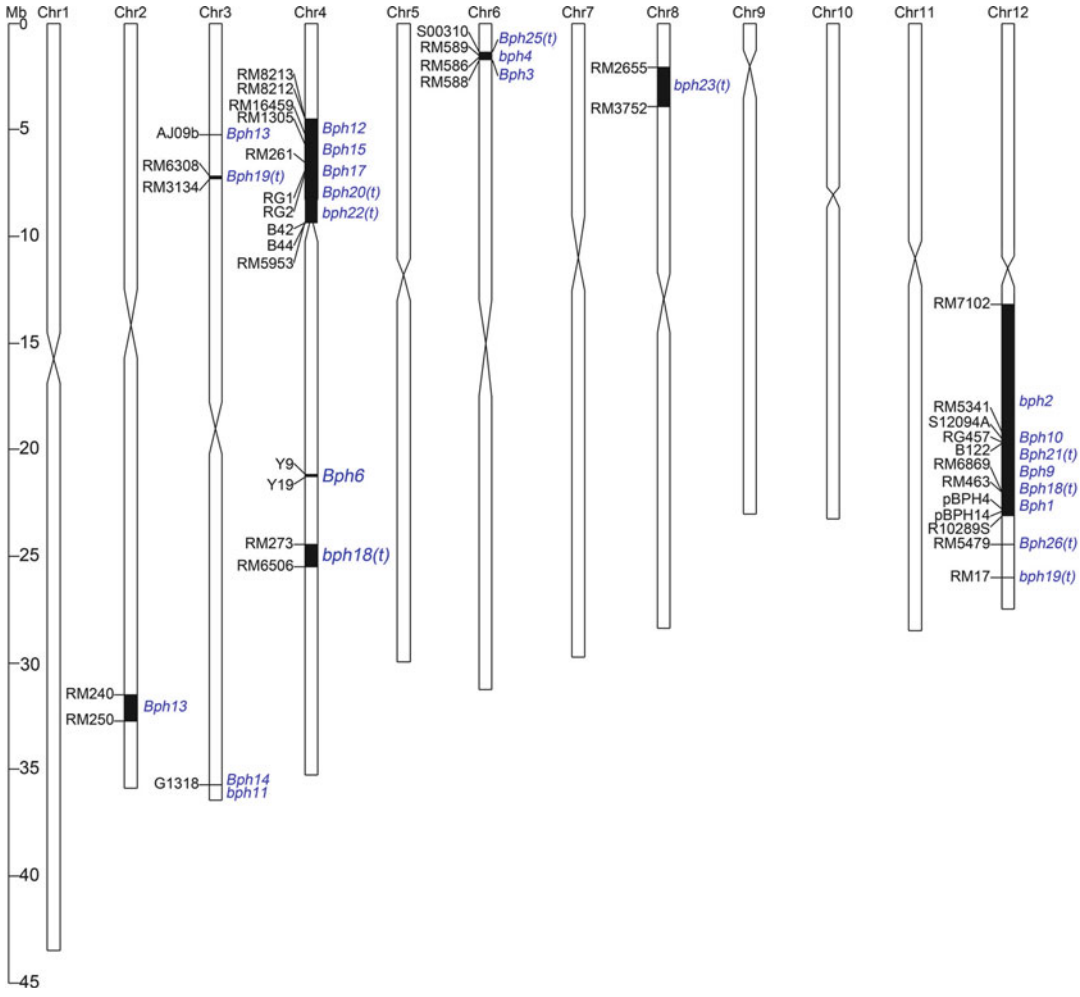

Fig. 12.1 Location of the brown planthopper resistance genes on the linkage map of rice. Marker names are listed on the left-hand side of the chromosomes. The positions of BPH resistance genes were integrated into the map based on the published data

Interestingly, 22 of the mapped BPH resistance genes are clustered on rice chromosomes (Fig. 12.1). In summary, eight BPH resistance genes (*Bph1*, *bph2*, *Bph9*, *Bph10*, *Bph18*(t), *bph19*(t), *Bph21*(t), and *Bph26*) cluster in one location that spans an estimated 13.7 Mb region on chromosome 12 based on the sequence of Nipponbare between markers RM7102 and RM17. Another seven genes cluster at two regions on chromosome 4: one that covers a region between markers RM8213 and RM5953 and contains *Bph12*(t), *Bph15*, *Bph17*, *Bph20*(t), and bph22(t) and another that includes *Bph6* and *bph18*(t) between markers Y9 and RM6506. Four genes (*bph11*, *Bph13*(t), *Bph14,* and *bph19*) have been mapped to chromosome 3. *Bph3*, *bph4,* and *Bph25*(*t*) are located on chromosome 6. It is well known that rice disease resistance genes also cluster in the same chromosome regions. It is possible that the clustered genes are different but tightly linked loci, that they represent different alleles at the same locus, or that they are the same allele but show differential reactions to different BPH biotypes. QTLs for BPH resistance have been detected in rice IR64 and B5, which play an important role in resistance durability.

5 Genes for Gall Midge Resistance

The gall midge *Orseolia oryzae* is a serious rice pest in South and Southeast Asia and parts of West Africa. Gall midge causes the leaf sheath to develop into an onion leaflike gall called an onion shoot or silver shoot. Damage results in plant stunting and reduces panicle number. Many sources of resistance have been identified. Inheritance studies have identified 11 gall midge resistance genes imparting resistance to different biotypes of Asian rice gall midge. Most of these genes, except *gm3*, *GM9,* and *GM10*, have been mapped on chromosomes. Tightly linked codominant SSR markers for Gm1 and Gm2 gall midge resistance genes have been developed [33]. Gm1 is resistant to gall midge biotypes 1, 3, 5, and 6, while Gm2 is resistant to biotypes 1, 2, and 5. Both genes lack resistance against biotype 4. PTB10, a land race from India containing Gm4, is resistant to biotypes 1, 2, 3, and 4 [92]. Recently, tightly linked SSR markers RM22550 and RM547 for Gm4 in PTB10 were identified [86]. pyramiding these resistance genes by marker-assisted selection (MAS) should be applicable for development of durable gall midge resistant varieties against multiple biotypes.

6 Genes for Small brown planthopper Resistance

The small brown planthopper (*Laodelphax striatellus*, SBPH) occurs in Japan, Korea, and China and is a vector of rice stripe virus and black-streaked dwarf virus, both serious rice diseases. Researches on genetics of SBPH resistance in rice are few. Through resistance screening, Duan et al. [20] detected 25 rice accessions with different levels of resistance to SBPH. Three QTLs conferring resistance to SBPH, named as *Qsbph2b*, *Qsbph3d*, and *Qsbph12*, were detected on chromosomes 2, 3, and 12 in cultivar Mudgo; the contribution of each locus is low [18]. Indica variety Kasalath contained several QTL alleles for SBPH resistance. Three QTLs conferring antixenosis against SBPH and two QTLs expressing antibiosis to SBPH were detected on chromosomes 2, 3, 8, and 11, respectively [19].

7 Genes for Whitebacked planthopper Resistance

The whitebacked planthopper (WBPH), *Sogatella furcifera* (Horvath), is a serious insect pest of rice. So far, nine major WBPH resistance genes and several QTLs have been reported. Among these genes, *Wbph1–Wbph5* were identified through classical genetic analysis. Later, *Wbph2* was linked with the marker RZ667 on chromosome 6, and the distance of RZ667 to *Wbph2* was 25.6 cM [75]. *Wbph6* detected in variety Guiyigu was mapped onto the short arm of chromosome 11 with a genetic distance of 21.2 cM to SSLP marker RM167 [69]. *Wbph7* and *Wbph8* introgressed from *O. officinalis* were mapped on the same location as BPH resistance genes *Bph14* and *Bph15* on chromosomes 3 and 4, respectively [124]. All these WBPH resistance genes confer seedling resistance to the insects. A gene ovicidal to WBPH designated as Ovc was identified in cultivar Asominori and mapped on rice chromosome 6 [135]. A number of QTLs for seedling resistance and for ovicide have been identified [11, 134].

8 Genes for Green Leafhopper Resistance

The Green leafhopper (GLH), *Nephotettix virescens*, is a serious pest in the tropics and subtropics, which causes yield losses by direct feeding as well as by acting as a vector for yellow dwarf and tungro diseases. Many resistant varieties have been identified. Genetic analysis has revealed 11 dominant and three recessive genes (*Glh1*, *Glh2*, *Glh3*, *glh4*, *Glh5*, *Glh6*, *Glh7*, *glh8*, *Glh9*, *glh10*, *Glh11*, *Glh12*, *Glh13*, and *Glh14*). Among them, *Glh1* in Pankhari 203, *Glh2* in ASD7, *Glh3* in IR8, *glh4* in Ptb8,

Glh5 in ASD8 and *O. rufipogon*, *Glh6* in TAPL796, and *Glh14* in ARC11554 have been mapped on chromosome 5, 11, 6, 3, 8, 5, and 4, respectively [8].

9 Genes for Green Rice Leafhopper Resistance

The green rice leafhopper (GRH), *Nephotettix cincticeps* Uhler, is a major insect pest of cultivated rice and is distributed mostly in temperate regions of East Asia [28]. Six major genes for GRH resistance have been identified and mapped on rice chromosomes. The *Grh5* was derived from wild species *O. rufipogon*. The *Grh6* was first identified from the Surinam cultivar SML17 in 1999 [123]. Latter, Fujita et al. [22] detected a GRH resistance gene in *O. nivara* at the same position as *Grh6* so they named it as Grh6-nivara. QTLs have also been identified in *O. rufipogon* and West African cultivar *O. glaberrima*.

10 Genes for Leaf Folder Resistance

The leaf folder *Cnaphalocrocis medinalis* is distributed throughout Asia. The larva rolls leaf by tying silk to the margins and feeds within the rolled leaf, removing the green layer. Damage usually occurs during the reproductive stage of plant and causes yield loss. In China, leaf folder is among the most serious insects of rice crop. Though resistant materials have been identified in cultivated rice and wild rice species, no major resistance gene has been reported. The Chinese variety CJ06 is resistant to leaf folder. A double haploid (DH) population of CJ06/TN1 was used to investigate the genetic basis for leaf folder resistance. QTL alleles increasing the resistance to leaf folder were detected from both CJ06 and TN1. The genetic effect of each locus is small; however, results showed that QTL pyramiding improved the leaf folder resistance remarkably [99].

11 Insect Resistance Gene Cloning and Functional Study in Rice

Among the insect resistance genes listed in Table 12.3, *Bph14* is the first one cloned through a map-based cloning approach [17]. Initially, the *Bph14* gene was identified as a major QTL in rice B5 [44]. A recombinant inbred line RI35 that carries only *Bph14* was selected and used to develop mapping populations. *Bph14* was localized within a 120 kb physical region on chromosome 3 flanked by the markers RM570 and G1318. Fine mapping with 5,000 F5 plants delimited the *Bph14* gene to a 34 kb region which contained two candidate genes, named as *Ra* and *Rb*. The candidate genes were transferred into the BPH-susceptible *indica* variety Kasalath, respectively, and the T2 families were examined for BPH resistance. Only the transgenic lines expressing *Ra* showed enhanced resistance upon BPH bioassay. *Bph14* gene encodes a coiled-coil nucleotide binding and leucine-rich repeat (CC-NB-LRR) protein. Another resistance gene, *Bph18*, was also cloned. This gene encodes a coiled-coil nucleotide binding site (CC-NBS) protein lacking LRR domain [52]. The results of *Bph14* and *Bph18* reveal the commonality in the mechanisms of plant defense against pathogens and rice plant hoppers.

Little is known about how rice perceives and interacts with the insect and functional genomics study on rice resistance to insects is just in its starting stage. Various molecular techniques, including suppression subtractive hybridization, cDNA array analysis, and proteomic and metabonomic approach, have been used to describe rice responses to BPH feeding and striped stem borer [41, 73, 128–131, 141, 142, 145]. When attacked by insects, plants can recognize specific elicitors or effectors (though for rice insects, the elicitors and effectors have not yet been identified), which activate various signaling pathways including jasmonic acid (JA), ethylene (ET), and salicylic acid (SA), that regulate signal transduction cascades in plant cells, leading to the activation and

modulation of defense-related genes [38, 68, 108]. Plants show varied responses to herbivores that are strongly correlated with the mode of herbivore feeding [30]. Rice response to BPH may be similar to those of plant–pathogen interactions. The transcriptome, proteome, and metabolome are reorganized in rice that attacked by BPH. Genes involved in plant defenses and macromolecule degradation were upregulated, whereas those involved in photosynthesis and cell growth were downregulated. Leaf senescence is most likely activated in the susceptible rice plants damaged by BPH. Immune response of rice to BPH might activate JA and SA signaling pathways and involve OsMPKs. Upstream genes involved in ET production are rapidly upregulated by BPH feeding, and ET emission is continuously enhanced thereafter until 72 h. In addition, transcript levels of genes associated with a JA-dependent pathway changed in rice plant after attacked by BPH [40]. In another study, increasing OsLOX1 activity and transcript level makes rice plant more resistant to BPH. Transformants with a lower level of OsLOX1 expression were less tolerant [128, 130]. Du et al. [17] compared *Bph14*-transgenic plants with the WT plants and found that the transcript levels of the SA synthesis-related genes were higher in the *Bph14*-transgenic plants but that of JA/ET-dependent pathway genes were substantially enhanced in the WT plants after BPH infestation. So, the *Bph14* may activate an SA-dependent resistance pathway after BPH feeding. These studies indicated that resistant plant and susceptible plant may activate different signal pathways in response to BPH feeding.

The JA pathway plays a central role in plant defense responses against chewing insects that cause extensive tissue damage and activate wound signaling pathways [55]. Transcription of gene involving in jasmonate signaling pathway was activated by stripe stem borer (SSB) feeding rapidly [120]. *OsNPR1* might be an early responding gene in herbivore-induced defense that negatively regulated the levels of JA and ET and plant resistance to a chewing herbivore [66]. The 13-lipoxygenase (13-LOX) is an enzyme in the biosynthesis of JA. Antisense suppression of 13-LOX (as-lox) reduced SSB-induced JA and trypsin protease inhibitor (TrypPI) levels, improved the larval performance of SBB and rice leaf folder, and increased the damage of rice plant caused by SSB and leaf folder larvae [144]. In contrast, 13-LOX-suppressed plant reduced the feeding and survival of the phloem-feeding insect BPH. These results imply that JA might play contrasting roles in rice resistance to chewing and phloem-feeding herbivores in rice.

12 Insect Resistance Mechanism Based on Physiological Function

Long-term coevolution of herbivorous insects and plants has led to the development of an array defenses that enable plants to protect themselves from herbivore attack. Generally, plants may employ three resistance mechanisms against insects with respect to physiological function. These mechanisms are *antixenosis*, reduction in colonization or oviposition; *antibiosis*, reduction in insect survival, growth rate, or reproduction after ingestion of host tissue; and *tolerance*, production of a crop of high quality and yield despite insect infestation [1]. Antixenosis, antibiosis, and tolerance result from independent genetic characters which work in combination.

Tolerance refers to the response of the plant to the insect. The main mechanism of tolerance is compensation. Rice plants, particularly high-tillering varieties, have an ability to compensate for damage by insect pests. For example, research has shown that more than 75 % of rice seedlings can be damaged by the whorl maggot without suffering yield loss [98]. This compensatory ability declines as the plant matures. Plant weight loss, leaf yellowing, and yield reduction are taken as tolerance indicators [91, 94]. The genetic basis for tolerance is not well elucidated.

Antixenosis and antibiosis refer to the response of the insect to the plant. The molecular

mechanism of antixenosis and antibiosis is not well understood. Some secondary metabolites, volatiles, and defense proteins, such as proteinase inhibitors, lectins, and various kinds of enzymes, decrease the attraction, growth, survival, and fecundity of insects. The plant hoppers of rice are piercing and sucking insects. BPH uses its stylet mouthparts to puncture and probe the epidermis, to penetrate the plant cell walls, subsequently to salivate into the cells, and to ingest the phloem sap. Sensory structures on the labial tip of BPH receive chemical stimuli during the exploration of the plant surface that exerts an antixenotic function and affects the host choice. Chemical cues originate from the alkanes and carbonyl compounds of the epicuticular wax. Higher ratio of long to short carbon chain in waxes is found in resistant cultivars [31, 133].

Mechanical barriers that inhibit phloem sap ingestion contribute to resistance. The time for ingesting phloem sap by BPH nymphs on the resistant plant is much less than on susceptible plant. The quantity of phloem sap ingested from the highly resistant B5 plant, which carries two resistance genes, was only one fortieth of that from susceptible plants. Hao et al. [30] further found that callose was deposited on the sieve plates in resistance rice that occludes the phloem and prevents BPH feeding. In the susceptible rice plants, callose-hydrolyzing enzymes were induced by BPH feeding, which decompose callose on the sieve plates and facilitate BPH feeding [17, 30, 131]. Reduced feeding of BPH on resistant plants was also attributed either to a lack of phagostimulants or to the presence of antifeedants [107]. Amino acid asparagine was considered to be a sucking stimulant, and its concentration is low in the resistant cultivar Mudgo [115]. The soluble silicic acid, oxalic acid, and sitosterol in resistant rice plants were identified as sucking inhibitors [56, 111]. A well-known defense component is trypsin proteinase inhibitor (PI), which is thought to play an important role in resistance to insect. The PI gene was upregulated in the rice plant challenged by BPH [17, 132]. In addition, the SSB feeding induced the accumulation of PIs in rice [120, 145].

For chewing insect of rice like stem borers, various plant morphological, anatomical, biochemical, and physiological factors have been suggested as imparting resistance [10]. Tight leaf sheaths were proved to prevent newly hatched larvae from feeding on the inner part of the leaf sheath before boring into the stem [125]. Antixenosis to stem borer oviposition is considered to be a biochemical nature. Oryzanone (*p*-methylacetophenone) attracts ovipositing moths and larvae of stem borer [79]. Allomones inhibit oviposition and adversely affect egg hatching, larval survival, and larval development of the SSB [46]. Silica content has also been associated with resistance to stem borers. Mandibles of larvae feeding on a cultivar with high silica content were worn down, and larvae with damaged mandibles have a lower feeding efficiency and suffer higher mortality compared with those feeding on rice with low silica content [16].

Volatiles are also the indirect defense of plant, which signal the location of insects on infested plants to parasitoids and predators [38]. Infestation by BPH and stem borer activated pathways to produce volatile organic compounds in rice that attract the parasitoid *A. nilaparvatae* and enhance the parasitism [76, 128, 130].

13 Breeding for Insect Resistant Rice

Growing resistant rice variety is an effective and environment-friendly way to control insect pests of rice. Zhang [143] proposed green super rice as a goal for rice breeding. Green super rice should possess resistances to multiple insects. Traditionally, breeding for insect resistance is based on phenotypic selection. A vast number of breeding materials are grown and evaluated for various agronomic characteristics, grain quality, multiple resistance, and tolerance. In such a way, IRRI has developed rice varieties resistant to BPH and GLH and moderately resistant to SSB [61]. In this process, growing the segregating populations and resistance evaluation is the most

laborious and technical part; thus, cooperation of breeders with entomologists is essential.

Application of molecular markers has made an impact on breeding process for insect resistance. MAS that targets the resistance gene includes a number of merits. The most successful applications of MAS in rice breeding have been those for major resistance genes to BPH, GM, and WBPH. Gene pyramiding has been increasingly recognized as an efficient approach to cope with the frequent infestations by BPH. The rice lines pyramided with two BPH resistance genes showed higher resistance and stronger antixenosis and antibiosis towards the BPH than the single gene introgression lines [64, 65, 67, 94]. Rice hybrids containing a single BPH resistance gene showed enhanced resistance compared to conventional hybrids, and pyramiding two genes provided even higher resistance [39]. Resistance of rice containing two or more resistance loci is considered to be durable. In China, male-sterile lines, restoring lines, and hybrid varieties incorporated with *Bph14* and *Bph15* are developed and released to the farmers.

Recent biotechnological interventions have opened up new opportunities for pest control by developing transgenic varieties with insecticidal genes, such as genes encoding the δ-endotoxin from *Bacillus thuringiensis* (Bt) and genes encoding snowdrop lectin (*Galanthus nivalis* agglutinin, GNA). A high level of resistance to stem borers has not been found in rice germplasm; Bt gene that is highly effective against lepidopteran insects is the first choice for controlling rice borers and leaf folders. Lectin is another important insecticidal gene resource due to its particular capacity to control rice sap-sucking (hemipteran) pests, which cannot be controlled by Bt toxins. Bioassay showed that transgenic GNA rice plants could reduce the growth, development, and fecundity of the infested pests [84, 97, 119]. However, resistance of GNA to rice plant hoppers and leafhoppers is not comparable to that of Bt to lepidopteran insects and to that of the resistance genes identified in rice germplasm.

14 Future Challenges and Perspectives

Insect pest is expected to be a major constraint for rice production in the future. Functional genomic understanding of rice–insect interaction will greatly accelerate development of resistant variety. Gene diversity is a security to counteract the virulence variation of insect. Identification of new source of resistance to insect in wild species germplasm from different countries particularly in hot spots will enhance insect resistance gene diversity in rice. Combinatory approaches of genetics and genomics will provide better understanding of gene function and the genetic and molecular basis for rice–insect interaction and coevolution.

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Abiotic Stress Resistance

13

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Abiotic stresses, such as drought, submergence, salinity, and low temperature, are major limiting factors for rice growth and production. All these stresses have very complex genetic bases, and both overlapping and distinct molecular and physiological mechanisms are involved in plant tolerance to different stresses. Our understanding of the molecular basis of plant response and adaptation to diverse abiotic stresses is mainly from the model plant Arabidopsis. Rice, one of the most important food crops worldwide, has been domesticated under relatively favorable environmental conditions and is extremely sensitive to abiotic stresses. In the last decade, numerous studies have been conducted on abiotic stress resistance of rice, including germplasm exploitation and breeding, genetics, genomics, and gene identification for stress resistance improvement. In this chapter, recent progress in genetic and genomic studies and characterization of stress-related genes in rice will be reviewed. This chapter will focus on drought stress, while the progress with regard to other stresses will be briefly reviewed.

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1 Drought

Drought causes major economic losses in crop production throughout the world. For example, global losses to drought of the two major cereal crops, rice and maize, are estimated to be more than US$5 billion annually. Drought has been, and continues to be, the single most devastating factor menacing food production and food security, especially in areas with inadequate agricultural water resources, and improving drought resistance is one of the major goals in developing "Green Super Rice" [222].

Drought resistance is a complex trait that involves numerous aspects of developmental, physiological, biochemical, and molecular adjustments. These include, for example, changes in root growth, stomata aperture, and osmotic adjustment; alterations in photosynthesis; and synthesis of protective proteins and antioxidants. Drought tolerance and drought avoidance, two major mechanisms for drought resistance in rice [220], have been extensively studied, while other mechanisms, such as drought escape and drought recovery, are seldom addressed. In this section, the genetic bases of drought resistance are reviewed, followed by progress of genomic studies and characterization of drought resistance-related functional genes.

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Table 13.1 QTL for root traits detected in different rice populations

Population parents	Population type	Traits	Growth condition	Measurement stage	Reference
IR58821/IR52561	RILs F_7	Root number (RN), penetration	66.7 % paraffin wax	65 days after sowing (DAS)	[4]
IAC165/Co39	RILs F_7	Maximum root length (MRL), root thickness, root dry weight (RDW)	Sandy loam soil (tube)	45 DAS	[25]
IR64/Azucena	DH	RN, root volume (RV), RDW	Sandy loam soil (tube)	65 DAS, 85 DAS	[57]
Akihikari/IRAT109	BC_1F_4	MRL, RDW	Growth medium (pot)	30 DAS	[60]
CT9993/IR62266	DH	MRL, RV, deep root weight (DRW)	Maahas clay soil (pot)	45 DAS	[82]
CT9993/IR62266	DH	MRL, RV, DRW	Maahas clay soil (pot)	45 DAS	[83]
IRAT109/Yuefu	DH	RN, root thickness (RT)	Lowland, upland	Maturity	[101]
		RN, RT, root/shoot ratio	PVC tube	Tillering	
Bala/Azucena	RILs F_6	MRL, DRW	Sub-soil (box)	Maturity	[116]
CT9993/IR62266	DH	Root penetration, RT	Wax-petrolatum	50 DAS	[226]
ZS97/Minghui63	RILs F_{10}	MRL, RDW, root/shoot ratio	Hydroponic	30 DAS	[206]
ZS97/IRAT109	RILs F_9	RV, DRW	Mixed soil (PVC tube)	Maturity	[220]
IR1552/Azucena	RILs F_{10}	Seminal root length (SRL)	Solution	15 DAS	[225]
IR1552/Azucena	RILs F_{10}	SRL, lateral root length, RN	Sandy soil (PVC tube)	14 DAS	[230]
Yuefu/IRAT109	RILs	RT, MRL, RN, RV, RDW, root fresh weight	Paddy soil (PVC tube)	Seeding, tillering, heading, grain filling, maturity	[148]

1.1 Genetics of Drought Resistance

1.1.1 Quantitative Trait Loci Mapping of Root Traits

Traits of the root system, such as root volume, maximum root length, root thickness, deep root rate, root penetration, and root/shoot ratio, are generally considered to be related to drought avoidance [24, 148]. Although root traits are very difficult to be measured when rice plants are grown in natural field conditions, a large number of quantitative trait loci (QTL) for root traits have been detected in rice grown in pots, hydraulic tanks, or polyvinyl chloride pipe (Table 13.1).

In general, most root traits have relatively low heritability, and the QTL mapping that results from the same genetic population are sensitive to the experimental condition or the developmental stage. For example, Yue et al. [219, 220] detected a total of 71 QTLs for root-related traits in 2 years by using a recombinant inbred line (RIL) population grown under two water regimes (well-watered and drought), but only six QTLs were detected in both years and nine QTLs were detected in both water regimes. Qu et al. [148] reported 86 QTLs involving six root traits measured at different developmental stages, but only 12 QTLs appeared in more than two stages.

QTLs for different root traits are often detected in the same chromosomal regions. For example, QTLs for six root traits were detected in the RM472–RM104 region on chromosome 1, and QTLs for nine root traits were detected in the RM470–RM317 region on chromosome 4 [219]. This phenomenon has been observed in most root QTL mapping studies [4, 101, 148, 226]. It remains to be determined whether this is due to a pleiotropic effect of a single QTL or to close linkage of different QTLs.

Even though most QTLs for root traits are affected by the genetic background of the mapping population and environment, some could be

detected in different populations or under different experimental conditions. In the RM231–RM175 region on chromosome 3, QTLs for basal root thickness were detected in two different populations [83, 101]. Zheng et al. [230] compared their QTL mapping results of maximum root length with the results from two other populations and found that three QTLs were commonly detected. Common QTLs detected in different populations were also mentioned in the studies for root penetration [226], basal root thickness, and deep root ratio [82]. These commonly detected QTLs in different populations may have high values in genetic improvement of root traits by marker-assisted section (MAS).

1.1.2 Genetics of Leaf Traits Related to Drought Resistance

Similar to root traits, many leaf-related traits are frequently used as criteria to evaluate drought resistance. These criteria include morphological (such as leaf rolling, dead leaf rate, stomatal size and density) and physiological (such as cuticular wax, abscisic acid [ABA] content, relative water content [RWC], water potential, canopy temperature) traits used for the evaluation of drought avoidance; physiological traits for drought tolerance (such as cell membrane stability, osmotic adjustment, dehydration tolerance, enzymatic activity related to reactive oxygen species scavenging, contents of proline, soluble sugar, and carotenoids); and other water use efficiency (WUE)-related traits [9]. The genetic basis of many leaf-related drought resistance traits have been studied by QTL mapping analyses (Table 13.2).

Among the drought avoidance-related criteria, ABA plays a very important role in drought response. Quarrie et al. [149] reported ten QTLs for ABA content in rice seedlings. Canopy temperature (CT) is generally considered useful in evaluating drought resistance at the population level. Yue et al. [219] and Liu et al. [111] detected 6 and 18 CT QTLs, respectively, but none of them was detected in both studies, indicating that CT may be strongly affected by the environment. Leaf rolling and dead leaf rate are empirical traits and can be easily scored. Yue et al. [219, 220] detected ten QTLs for leaf rolling and ten QTLs for dead leaf rate in 2 years. A few QTL for leaf water potential were reported in two studies [111, 147], but none of them was detected in both studies. Similarly, a few QTLs for RWC were reported by Price et al. [143] and Babu et al. [10], but only one was detected in both studies. For stomata density and size, 10 and 4 QTLs were detected, respectively [96], and one of the stomata density QTLs was very close to a stomata density QTL reported by Ishimaru et al. [71].

Genetic analyses of a few drought tolerance-related physiological traits, including cell-membrane stability (CMS), dehydration tolerance, osmotic adjustment (OA), have been reported. Nine QTLs were mapped for CMS in the population derived from CT9993/IR62266 [180], and five QTLs for OA were detected in the same population [226]. In the vicinity of the CMS QTL on chromosome 8 (interval G2312–R139A), QTL was detected for OA [105, 153, 226] and dehydration tolerance [105, 226]. More interestingly, this QTL has synteny with the QTL of OA in wheat [105] and barley [178], suggesting that this QTL may be conserved for drought resistance in cereals.

WUE is a generally acceptable criterion for evaluation of drought resistance but it is also difficult to measure. Five and six WUE QTLs were detected by Ishimaru et al. [70] and Xu et al. [208] in the same backcross population (Nipponbare/Kasalath), but none was commonly detected. Another two studies reported nine [143] and seven [175] QTLs related to WUE, and only three QTL had close positions in the maps.

1.1.3 Validation and Application of Drought Resistance QTL

Although a large number of QTLs have been reported for numerous drought resistance-related traits using primary populations, very few QTLs have been validated for their authenticity and no drought resistance QTL gene has been cloned.

Currently, meta-analysis and advanced populations are major approaches to validate QTL. Meta-analysis is based on consensus mapping that integrates different QTL maps for the same

Table 13.2 QTL for leaf traits detected in different rice populations

Trait	Parents	Population	No. QTL	Reference
ABA content	IR20/63-83	F_2	10	[149]
CT	ZS97/IRAT109	RILs F_9	6	[219]
	ZS97/IRAT109	RILs F_9	18	[111]
	CT9993/IR62266	DH	1	[10]
CMS	CT9993/IR62266	DH	9	[180]
DeT	Mor/CO39	RIL F_7	5	[105]
Drying leaves	ZS97/IRAT109	RILs F_9	4	[219]
	ZS97/IRAT109	RILs F_9	6	[220]
	Bala/Azucena	RILs F_6	11	[144]
	CT9993/IR62266	DH	3	[10]
Leaf rolling	ZS97/IRAT109	RILs F_9	6	[219]
	ZS97/IRAT109	RILs F_9	4	[220]
	Bala/Azucena	RILs F_6	5	[144]
	IR64/Azucena	DH	1	[57]
	Nipponbare/Kasalath	BC_1F_7	1	[71]
	CT9993/IR62266	DH	3	[10]
LWP	Yuefu/IRAT109	RILs	6	[147]
	ZS97/IRAT109	RILs F_9	8	[111]
OA	CT9993/IR62266	DH	5	[226]
	IR622/IR600	BC_3F_3	14	[153]
	Mor/CO39	RIL F_7	2	[105]
RWC	Bala/Azucena	RILs F_6	8	[144]
	CT9993/IR62266	DH	2	[10]
Stomatal density	Nipponbare/Kasalath	BC_1F_7	3	[70]
	IR69093/IR72	RILs	10	[96]
Stomatal size	IR69093/IR72	RILs	4	[96]
WUE	Nipponbare/Kasalath	BC_1F_5	5	[208]
	Nipponbare/Kasalath	BC_1F_7	6	[71]
	Bala/Azucena	RILs F_6	9	[143]
	Milyang 23/Akihihari	RILs	7	[175]
	ZS97/IRAT109	RILs F_9	4	[231]

CT canopy temperature, *CMS* cell-membrane stability, *DeT* dehydration tolerance, *LWP* leaf water potential, *OA* osmotic adjustment, *RWC* relative water content, *WUE* water use efficiency

or similar traits or in the same population under different environments to statistically identify authentic QTL or meta-QTL [7, 51, 186]. Meta-analysis can help distinguish pleiotropic QTL from tightly linked QTL related to different traits and shorten the interval length of the target [23, 87]. For example, Courtois et al. [24] performed a meta-analysis of 675 QTLs that had been reported for 29 root-related traits and found that these QTLs are mainly distributed in six intervals. The QTL for maximum root length can be shortened to a 20-kb region by the meta-analysis, in which only three genes encoding F-box domain proteins are predicted. Khowaja et al. [87] performed a similar meta-analysis of 165 QTLs for drought avoidance traits and identified a meta-QTL on chromosome 5 controlling drought avoidance, leaf shape, and biomass.

Although meta-analysis can increase the authenticity of putative QTLs detected in the primary populations, the QTLs still need to be validated by using advanced populations or near isogenic lines (NILs). So far, only a few drought resistance-related QTLs have been selected for validation (Table 13.3). Some of the QTL have been used in breeding programs by MAS.

Table 13.3 Evaluation of QTL for drought resistance

Trait	QTL name or location	Advanced population	Reference
Stele transversal area	*Sta1*	$BC_2F_{3:4}$	[183]
Deeper rooting	*Dro1*	BC_2F_3	[184]
Root thickness	*Brt4*	BC_1F_2, F_3	[108]
Root length	*qRL6.1*	NIL	[134]
Root (length, volume, etc.)	4 QTL on Chr. 1, 2, 7, 9	NIL	[165]
Root (length, volume, etc.)	4 QTL on Chr. 2, 7, 9, 11	NIL	[170]
Root volume	*qFSR4*	NIL	[30]
Yield under drought	*qtl12.1*	$F_{3:4-8}$	[13]

Four QTLs for root traits from Azucena were introduced into IR64 and some of the introgression lines showed the expected improvement of root traits [165]. In another effort, four QTLs for root traits from Azucena were introduced into Kalinga III, and one line containing the QTL (interval RM242–RM201 on chromosome 9) showed very significant increase of root depth [170], and four lines showed increases in grain yield and biomass in the field [171]. These efforts led to the development of new rice variety Birsa Vikas Dhan 111 (PY 84).

1.2 Genomics of Drought Resistance

Genome-wide association analysis, forward mutant screening, and transcriptomics (occasionally in association with proteomics and metabolomics) are widely adopted genomic approaches to reveal genetic and molecular bases of target traits in many organisms. Because of the complex genetic bases and diverse mechanisms for drought resistance, genome-wide association analysis and forward mutant screening approaches have seldom been applied in genomic studies of drought resistance in rice.

Quite a few studies have reported the transcriptomic change of rice after drought stress. By using a 70-mer oligomer microarray with 36,926 unique genes or gene models, global genome expression profiles of rice in response to drought and high-salinity stresses were surveyed in various tissues [233]. By using Affymetrix 57K gene chip, Ray et al. [151] found that 1,563 and 1,746 genes were up- and down-regulated, respectively, in 1-week-old seedlings of *indica* rice IR64 after acute water-deficit stress. In another analysis using the Affymetrix gene chip, 5,284 genes were found to be differentially expressed under acute drought stress, among which 261 transcription factor genes were differentially regulated [192]. However, by applying gradual, long-term drought stress to four rice cultivars with differing drought resistance, only 413 and 245 genes were found to be significantly induced and suppressed, respectively, by using a 20K oligonucleotide microarray [28]. In an effort to amalgamate data across laboratories, Ray et al. [151] identified 5,611 differentially expressing genes under water-deficit stress conditions in six vegetative stages and one reproductive stage of development in rice.

It can be estimated that approximately 8–10 % of rice genes are responsive to drought stress. Gene ontology (GO) analysis of reported genes and unpublished expression profiling data suggest that the drought-responsive genes encode diverse proteins that can be classified as regulatory and functional proteins. Regulatory proteins include, but are not limited to transcription factors (TFs), protein kinases, protein phosphates, phospholipase, signal reception and transduction, and producers of secondary messengers. Functional proteins are key enzymes in the biosynthesis of osmoprotectants like polyols and sugars, amino acid and quaternary ammonium compounds, cell wall loosening and structural components, cholesterol and very long chain fatty acids, phytohormones like ABA, and secondary metabolites. Among the drought-responsive genes, TFs are often overrepresented, which is especially true for TF families such as AP2/EREBP, zinc finger, bZIP, NAC, and MYB. In addition to protein-encoded genes, a significant proportion of miRNAs and siRNAs have been identified as being drought-responsive by using high-throughput deep sequencing [77], miRNA microarray [232], and stem-loop qPCR [166] approaches.

Genome-wide gene expression profiling has also been used to compare the transcriptomic changes of rice varieties with differential responses to drought. Degenkolbe et al. [28] investigated the physiological and gene expression responses of drought-tolerant (IR57311 and LC-93-4) and drought-sensitive (Nipponbare and Taipei 309) rice cultivars subjected to drought stress. In general, significantly more genes were regulated in the sensitive cultivars than in the tolerant cultivars, and some of these differentially responsive genes co-localized with published QTL regions for drought resistance. Lenka et al. [99] compared the drought stress-responsive transcriptome changes between drought-tolerant landraces/genotype N22 and the high-yielding but drought-susceptible rice variety IR64. GO analysis suggested that drought tolerance of N22 may be attributable to the enhanced expression of several enzyme-encoding genes and up-regulation of the alpha-linolenic acid metabolic pathway. Drought susceptibility of IR64 was attributable to significant down-regulation of regulatory components and starch and sucrose metabolism that confers drought tolerance. The differentially regulated genes provide a very useful resource for the functional dissection of the molecular mechanism in rice responding to environmental stress [192].

In addition to the genome-wide expression profiling of rice under drought stress, profiling of drought-responsive gene expression has been conducted at the gene family level. The reported gene families in rice with drought-stressed expression profiles include regulatory gene families, such as AP2/EREBP [164], NAC (NAM, ATAF, and CUC) [35], WRKY [150], HD-ZIP [2], TIFY [216], calcineurin B-like protein interacting protein kinase (CIPK) [200], receptor-like kinase [50], XHS domain-containing family [146], ribosome-inactivating proteins [79], and functional protein- or enzyme-encoding gene families such as the aldehyde dehydrogenase family [48] and the BURP domain-containing family [29]. The expression profiles of specific gene families also provide useful information for further functional analysis of the family members.

Recently, some efforts have been made to integrate expression profiling with proteomic and metabolomic analyses in rice under drought stress. For example, Shu et al. [169] performed proteomic analysis to determine the response of rice plant seedlings to drought-induced stress and identified 60 protein spots significantly altered by drought. This group also performed cDNA microarray and GC–MS analysis and identified 4,756 differentially expressed mRNAs and 37 differentially expressed metabolites. Integration analyses of the "omics" data revealed that increased energy consumption from storage substances occurred during drought, and increased expression of the enzymes involved in anabolic pathways corresponded with an increase in the level of six amino acids [169]. Another proteomic survey performed specifically in the anther of two rice genotypes revealed that drought-tolerant genotype Moroberekan possessed better recovery capability following drought and rewatering at the anther proteome level than the drought-sensitive genotype IR64 [107].

1.3 Characterization of Drought Resistance-Related Genes

Expression profiling analyses suggest that thousands of genes are responsive to drought stress. However, only a small portion of these genes have been characterized for their functions or effects on drought resistance. Genes were selected for functional analysis based on their responsiveness to drought, sequence similarity to known stress-related genes, or drought-sensitive or -tolerant mutants. The characterized genes encode proteins with very diverse functions. For ease of reference, the representative genes in each category are listed in Table 13.4 along with genetic, molecular, and/or physiological evidence for drought resistance.

Stress signal perception is the first step in a plant's response to drought. However, very few genes encoding receptors of drought stress signal or ABA have been characterized in rice. Among the functionally characterized rice genes that are involved in downstream of stress signaling perception, genes encoding protein kinases, transcription factors, and protein degradation or

Table 13.4 Categories of rice genes functioning in drought resistance

Functional category	Genes[a]	Protein function	Effect on DR; genetic materials; testing condition; supporting measurements or molecular evidence[b]	References
Protein kinase (*PK*)				
MAPK cascade	*DSM1*	Raf-like MAPKKK	P; KM/OE; GH/FD; ↓ ROS scavenging, EL, Chl in mutant	[132, 204]
	OsMAPK5	MAPK	P; OE/RI; GH; ↑ sensitivity to disease	
CIPK	*OsCIPK12*	CBL-interacting PK	P; OE; GH	[200]
CDPK	*OsCDPK7*	Calcium-dependent PK	P; OE; GH	[155]
Other kinase	*OsSIK1*	Receptor-like kinase	P; OE/RI/KM; GH; ↑ ROS scavenging enzyme activity	[91, 138]
	OsGSK1	Orthologue of BIN2	N; MT; GH	
Protein phosphatase				
Secondary messenger producer	*DSM3*	Inositol 1,3,4-trisphosphate 5/6-kinase	N/P (optimal expression required); OE/RI; GH/FD; ↓ IP3	[31]
Transcription factors				
CBF/DREB/AP2/ERF	*SUB1A*	Ethylene response factor	P; isogenic line/OE; GH/FD; MA, ↑ AS, ↓ LWR	[19, 26, 43, 136, 190]
	OsAP37	APETELA2 (AP2)	P: OE; GH/FD; ↑ yield under drought	
	OsDREB2A	DREB subfamily	P; OE (inducible promoter); GH	
	OsDREB1G,2B	DREB subfamily	P; OE; GH	
	OsDREB1F	DREB subfamily	P; OE; GH	
bZIP	*bZIP23*	Basic leucine zipper	P; OE/KM; GH; ↑ AS, MA (stress-related genes ↑)	[5, 112, 176, 201]
	bZIP46CA1		P; OE/KM; GH; stress-related genes ↑	
	bZIP72		P; OE; GH; ↑ AS, LEA genes ↑	
	OsABF1		P; MT; GH	
NAC	*SNAC1*	Stress-responsive NAC	P; OE/RI; FD/GH; ↑ SC, ↑ LWR, ↑ AS, ↑ WUE, MA	[62, 76, 126, 228]
	OsNAC10	NAC family	P; OE (by root promoter); GH; ↑ root thickness, ↑ yield,	
	ONAC045	NAC family	P; OE; GH; (some stress-related genes ↑)	
	OsNAC6	NAC family	P (dehydration); OE; GH; MA (↑ peroxidase gene)	
MYB	*OsMYB3R-2*	R2R3 MYB	P; OE (in Arabidopsis); GH; ↑ freezing and salt stresses	[27]
Zinc finger	*DST*	C2H2 zinc finger	N; KM; GH; ↑ SC and stomatal density in mutant by ↑ H_2O_2	[64, 84, 110, 205]
	ZFP252	TFIIIA-type zinc finger	P; OE/RI; GH; ↑ proline and sugar	
	OsiSAP8	A20/AN1 type ZF	P; OE; GH	
	OsCOIN	RING finger	P; OE; GH; ↑ proline	
MYC/bHLH	*OsbHLH148*	bHLH TF	P; OE; GH; MA (OsDREB and OsJAZ genes ↑), ↔ OsJAZ1	[162]
Other TF	*OsWRKY45*	WRKY family	N; KM/OE; GH;↑ AS	[177]
Protein degradation	*OsDIS1*	E3 ligase	N; OE/RI; GH; MA, ↔ OsNek6 (protein kinase)	[11, 49, 131, 140, 214]
	OsSDIR1	E3 ligase	P;OE;GH; ↑ SC, ↓ LWR	
	OsRDCP1	RING domain-containing	P; OE/KM	
	OsDSG1	E3 ligase	N; OE/KM; GH; ↑ Chl	
	MAIF1	F-box domain	N; OE; GH; ↓ AS	

(continued)

Table 13.4 (continued)

Functional category	Genes[a]	Protein function	Effect on DR; genetic materials; testing condition; supporting measurements or molecular evidence[b]	References
Protein modification	*SQS1*	Farnesyltransferase/ squalene synthase	N; RI; GH; ↑ yield, stomatal conductance, RWC in RI	[118]
Other nuclear proteins	*OsSKIPa*	Ski-interaction protein	N; OE/RI; GH; ↑ cell viability, MA, ↔ diverse proteins	[61, 78]
	OsRIP18	Ribosome-inactivating protein	P; OE; GH; MA	
Ca^{2+} sensor	*OsMSR2*	Calmodulin-like	P; OE (in Arabidopsis); GH; ↑ AS	[207]
ABA metabolism	*DSM2*	Carotene hydroxylase	P; OE/KM; GH/FD; ↑ xanthophylls and NPQ, ↑ stress genes	[32]
Metabolism of other hormones	*TLD1*	OsGH3.13 (IAA-AA synthetase)	P; activation mutant; GH; altered plant architecture, ↑ *LEA* genes	[224]
Cuticular wax synthesis	*OsGL1-1*	GL1 homologues	P; KM; GH; ↓ cuticular wax (in mutant)	[72, 145]
	OsGL1-2		P; OE/KM; GH/FD; ↓ cuticular wax (in mutant)	
Dehydrin	*OsLEA3-1*	LEA protein	P: OE; FD; ↑ yield under drought in the field (2 year repeats)	[202]
Osmotic adjustment	*OsTPS1*	Trehalose-6-phosphate synthase	P; OE; GH; ↑ trehalose and proline	[102]
Aquaporins	*RWC3*	Water channel protein	P; OE; GH; ↑ root osmotic hydraulic conductivity, leaf WP	[104]
Other transporters	*OsABCG31*	ABC transporter	P; KM; GH; ↑ LWR in mutant	[18]
Other defense-related proteins	*OsMT1a*	Type 1 metallothionein	P; OE; GH; ↑ Zn^{2+}; ↑ antioxidant enzyme activities	[66, 160, 193, 215]
	sHSP17.7	Small heat shock protein	P; OE; GH; ↑ recovery growth	
	OCPI1	Chymotrypsin inhibitor	P; OE; GH;↑ chymotrypsin-inhibitor activity	
	OsPR4a	Pathogenesis-related 4	P; OE; GH/FD	

Abbreviations for additional traits and measurements: *MA* microarray analysis of OE or mutant, *AS* ABA sensitivity, *SC* stomatal closure, *LWR* leaf water-loss rate, *WP* water potential, *WUE* water use efficiency, *ROS* re-active oxygen species, *EL* electrolyte leakage, *Sen* senescence, *Chl* chlorophyll content
↑ (Increased) and ↓ (decreased): change in OE unless specified; ↔: protein interaction
[a]Due to the limitation of the chapter, only representative or well-characterized genes were listed
[b]Effect on DR (drought resistance): *P* positive, *N* negative. Genetic materials: *OE* overexpression (in rice unless specified), *RI* RAN interference, *KM* knockout mutant. Testing conditions: *GH* growth chamber or green house (with plants grown in pots or medium), *FD* field, *VS* vegetative or seedling stage, *RS* reproductive or flowering stage

modification are overrepresented. Here, a few genes that have been well-characterized for their roles in drought resistance are briefly introduced.

Several genes encoding diverse protein kinases, such as mitogen-activated protein kinase (MAPK) cascade, CIPK, calcium-dependent protein kinase (CDPK or CPK), and receptor-like kinase, have been reported to have critical roles in drought resistance (see details in Table 13.4). OsMAPK5 is the first functionally characterized member of the rice MAPK cascade, and the *OsMAPK5* gene positively regulates abiotic stresses (including drought) but negatively regulates biotic stresses [204]. In the search to find the upstream component of the MAPK cascade (such as MAPK kinase [MAPKK] and MAPK kinase kinase [MAPKKK]), a Raf-like MAPKKK gene *DSM1* was recently reported to play an essential role in drought responses, potentially through regulating ROS scavenging in rice [132]. However, the substrates or the downstream components of these protein kinases remain to be identified.

Among the transcription factor genes that function in drought resistance, members from

DREB (drought-responsive element binding protein) or AP2/ERF genes from many plant species have been identified to be effective in improving drought tolerance, including at least five *DREB* genes in rice (Table 13.4). Similarly, many bZIP genes homologous to *ABI5*, a key regulator of ABA signaling in Arabidopsis, have been reported in diverse plant species and include *OsbZIP23* [201], *OsbZIP46* [176], *OsbZIP72* [112], and *OsABP1* [5] from rice.

SNAC1 is a transcription factor belonging to the NAC (NAM, ATAF, and CUC) family. Transgenic rice overexpressing the *SNAC1* gene showed significantly enhanced drought resistance (22–34 % higher seed setting than the control) in the field under severe drought-stress conditions at the reproductive stage, while showing no phenotypic changes or yield penalty [62]. The SNAC1 transgenic rice lost water more slowly by closing more stomatal pores, and maintained turgor pressure at a significantly lower level of RWC than the wild type. DNA chip analysis revealed that more than 150 genes were up-regulated (>2.1-fold) in the *SNAC1*-overexpressing rice plants. Another three members of this family, OsNAC6 [126], ONAC045 [228], and OsNAC10 [76], have been suggested to have positive roles in improving drought or dehydration tolerance in rice.

At least five zinc finger genes from rice have been reported to have roles in drought tolerance (Table 13.4). The *DST* gene deserves special notice because it negatively regulates drought resistance [64], while the other zinc finger genes show positive effects on the stress resistance. DST (drought and salt tolerance) is a previously unknown zinc finger transcription factor that negatively regulates stomatal closure by directly modulating expression of genes related to H_2O_2 homeostasis. Loss of DST function causes increased stomatal closure and reduced stomatal density, consequently resulting in enhanced drought tolerance in rice [64].

A few nuclear proteins such as OsSKIPa [61] and OsRIP18 [78] with regulatory roles in stress tolerance of rice have also been identified. OsSKIPa is a rice homolog of human SKIP (Ski-interacting protein) that can complement the lethal defect of the knock-out mutant of SKIP homolog in yeast and positively modulate cell viability and stress tolerance in rice [61]. Transgenic rice overexpressing *OsSKIPa* showed significantly improved drought resistance and significantly increased ROS-scavenging ability and transcript levels of many stress-related genes. Interestingly, OsSKIPa can interact with more than 30 different proteins and most of these proteins have no matches with the reported SKIP-interacting proteins in animals and yeast [61].

Quite a few genes encoding E3-ligase, RING-domain-containing protein, or F-box protein, which are involved in protein degradation, have been identified with either positive (such as *OsSDIR1*, *OsRDCP1*) or negative (such as *OsDIS1*, *OsDSG1*, and *MAIF1*) roles in regulating drought resistance in rice (Table 13.4). Recently, a gene (*OsSQS1*) encoding a putative farnesyltransferase/squalene synthase was reported to have a positive role in drought resistance [118], suggesting that protein modification is also involved in the regulation of drought resistance.

In addition to the genes with regulatory functions that have already been described, a large number of genes encoding functional proteins have been claimed to have a positive effect on drought resistance in rice. The functional categories of these genes include ABA metabolism, metabolism of other phytohormones, cuticular wax synthesis, osmotic adjustment-related, aquaporins, transporters, and defense-related proteins (Table 13.4). With the advances of genomic studies and functional analyses of more candidate genes, it can be expected that an increasing number of genes belonging to these categories or to new categories will be identified as affecting drought resistance in rice.

2 Submergence Stress

Sudden or seasonal flooding can cause serious damage to rice growth and substantially reduce yield. Seasonal flooding regularly affects about 15 million hectares of rice-growing areas, mainly in South and Southeast Asia, and result in the economic loss of up to US$1 billion annually [154].

There are two different types of flooding, deepwater and flash, based on the strength and

duration of rainfall and geographical features. Deepwater flooding is a large-scale flooding lasting for a prolonged period, even for several months, with water level of a few to several meters, while flash flooding occurs suddenly on a relative small scale and lasts for a short period of time. In flood-prone areas, traditional local varieties called deepwater rice display a remarkable capacity for flooding-induced stalk elongation of up to 25 cm/day, and thus avoid submergence stress [12]. Submergence tolerance is another mechanism for rice to survive flooding; for example, by avoiding energy consumption associated with plant elongation [86, 179]. Both mechanisms (avoidance and tolerance) involve morphological and physiological responses and adaptations that are generally governed by many genes or QTL [74, 179]. In recent years, significant progress has been made in unveiling the genetic and molecular bases of submergence tolerance.

2.1 Deepwater Flood Tolerance

The deepwater rice can adapt to flooding by dramatically elongating leaves and internodes, with daily height increases of 20–25 cm, thus keeping the top leaves above the water surface for gas exchange and photosynthesis [187]. Such rapid growth in response to flooding involves a unique biological and environmental adaptation in deepwater rice. Currently, the genetic basis of deepwater responses has been revealed by using QTL analysis [127, 229] and cloning of key genes such as *SNORKEL1* and *SNORKEL2* that encode ethylene responsive factor (ERF)-type transcription factors to control underwater shoot elongation [55]. Expressions of *SNORKEL* genes are significantly increased in submerged stems and induced by ethylene treatment. Overexpression of *SNORKEL* genes in non-deepwater rice results in internode elongation even in dry conditions [55].

2.2 Flash Flood Tolerance

In contrast to the flood avoidance mechanism of deepwater rice, submergence-tolerant rice shows stunted growth, which allows it to survive submergence for a few weeks by avoiding energy consumption, thus conserving energy to restart growth after the flood has receded [54].

A major submergence-tolerance QTL (*Sub1*) on chromosome 9 explains about 70 % of the phenotypic variation in submergence-tolerant rice [210]. The *Sub1* region of submergence-tolerant varieties contains a variable cluster of three similar genes, *Sub1A*, *Sub1B*, and *Sub1C*, encoding ERF-type transcription factors. Submergence-intolerant varieties possess *Sub1B* and *Sub1C*, with lost or loss of function of *Sub1A*. Introducing Sub1A into submergence-intolerant varieties increases submergence tolerance [42, 209]. Sub1A negatively regulates the expression of the genes encoding enzymes for starch and sucrose digestion to reduce energy consumption during submergence and positively regulates the expression of alcohol dehydrogenase (ADH) genes and pyruvate decarboxylase (PDC) genes, thus promoting acclimation to hypoxic conditions [42]. SUB1A limits ethylene-driven shoot elongation by minimizing the decline of GA-signaling repressor SLENDER RICE-1 (SLR1) and SLR LIKE-1 (SLRL1) proteins in submerged shoots [41]. Furthermore, SUB1A suppresses ethylene synthesis and expression of cell wall loosening expansin genes under submergence [42]. Recent transcriptome profiling analysis suggests that SUB1A regulates multiple pathways associated with growth, metabolism of starch and sucrose, and stress endurance. With all data taken together, the *Sub1A* gene in specific rice accessions functions by minimizing energy utilization during submergence to prolong underwater survival [80].

Strikingly, all the key genes identified so far for flood tolerance (*SUB1A*) and avoidance (*SNORKEL*s) encode ERF-type transcription factors that are involved in the regulation of the ethylene signal transduction pathway, and GA and ethylene can trigger antithetical outcomes—promotion of SNORKEL or suppression of SUB1A [55, 142]. However, the precise mechanism by which rice responds and adapts to flood or submergence remains largely unknown. Nevertheless, the introduction of *SNORKEL* or

SUB1A into high-yielding varieties can improve the quality and quantity of rice produced in flood-prone marginal farmlands [163].

3 Salt Stress

Increasing soil salinization of limited farm land is becoming a serious global threat to sustained agricultural production. Plants adapt to salinity stress mainly by three distinct mechanisms: osmotic stress tolerance, Na^+ or Cl^- exclusion, and the tolerance of tissue to accumulated Na^+ or Cl^- [124]. Because of the complexity of the salt response and diversity of the tolerance mechanism in plants, the precise mechanism of salinity tolerance remains largely unknown in crops. Owing to the fast development of genetic and genomic resources, researchers have conducted QTL mapping, expression profiling, and characterization of genes related to salt tolerance in rice in the last decades.

By using an F_2 and an equivalent F_3 population, Lin et al. [106] detected three QTLs for survival days of seedlings, three QTLs for shoot-related traits, and five QTLs for root traits under salt stress. Subsequently, a gene *SKC1* for salt tolerance QTL (Saltol) on chromosome 1 was cloned by this group. SKC1 plays an important role in ion homeostasis during salinity stress, and overexpression of this gene in rice can significantly increase salt tolerance [152]. A few other groups also reported salt tolerance-related QTLs. Lee et al. [97] evaluated salt tolerance at the young seedling stage of rice by using an RIL population and detected two QTLs, qST1 and qST3, conferring salt tolerance. Zang et al. [221] identified 13 QTLs for survival days of seedlings, score of salt toxicity of leaves, shoot K^+ concentration, and shoot Na^+ concentration at the seedling stage and 22 QTLs underlying fresh weight of shoots, tiller number per plant, and plant height at the tillering stage. Pandit et al. [139] used salt ion concentrations in different tissues and salt stress susceptibility index for spikelet fertility, grain weight, and grain yield as criteria and detected eight significant QTLs for the salt ion concentrations and one QTL for the susceptibility index.

Meanwhile, a variety of ways were adopted to identify salt stress-responsive genes in rice. He et al. [56] constructed a cDNA library with the salt-tolerant rice cultivar Lansheng and identified 30 genes displaying altered expression levels following treatment with 150 mM NaCl. These genes were classified into five categories, including photosynthesis, transport, metabolism, stress resistance, and the other functions. In another study, subtractive cDNA libraries were constructed by using a salt-sensitive *indica* rice, IR64, and a salt-tolerant variety, Pokkali, and a total of 1,194 salinity-regulated cDNAs were identified [94]. DNA chip and cDNA microarray were also used to compare transcriptomic changes of salt-tolerant and salt-sensitive genotypes [16, 188]. Chitteti and Peng [22] investigated the differential phosphoproteome of rice under salt stress and identified 17 up-regulated and 11 down-regulated putative phosphoproteins and 31 differentially regulated proteins by salt stress using SYPRO Ruby stain. Nohzadeh Malakshah et al. [133] monitored the changes in abundance of plasma membrane-associated protein in response to salinity through a subcellular proteomics approach, and 24 differentially expressed proteins were characterized. These profiling results provided valuable information to reveal the molecular basis of salinity tolerance in rice.

Numerous genes functioning in salt tolerance have also been identified in rice. Many genes with functions in producing osmoprotectants, such as sugar, proline, and glybetaine, have been identified and can effectively increase salt tolerance of transgenic rice via the osmotic adjustment pathway [14, 69, 156]. Furthermore, many genes involved in regulating ion homeostasis have also been confirmed to play crucial roles in enhancing rice salt tolerance. OsNHX1 is the first identified vacuolar Na^+/H^+ antiporter on the tonoplasts in rice, and it plays important roles in compartmentalizing Na^+ and K^+ in the cytoplasm into vacuoles to increase salt tolerance [45, 46]. Four additional NHX-type antiporter genes in rice (*OsNHX2* through *OsNHX5*) were later identified, and the members of this gene family were also proven to play important roles in the compartmentalization of the cytosolic Na^+ and K^+

into vacuoles [44]. A Na^+ transporter (OsHKT1), a Na^+- and K^+-coupled transporter (OsHKT2), and OsVHA were suggested to act in harmony in the salt-tolerant rice Pokkali, and subsequent studies indicated that OsHKT1 and OsHKT2 together with OsVHA might confer salt tolerance by maintaining a low cytosolic Na^+ level and a correct ratio of cytosolic Na^+ to K^+ [59, 81]. The *SKC1* gene, which was isolated by map-based cloning, encodes an HKT-type transporter functioning as a Na^+-selective transporter regulating K^+/Na^+ homeostasis under salt stress [152]. The rice homolog of Shaker family K^+ channel KAT1 (OsKAT1) is involved in salt tolerance of rice through cooperation with other K^+ channels by participating in the maintenance of cytosolic cation homeostasis during salt stress [135]. In addition to these transporters, many regulatory proteins, including protein kinases, phospholipases, calmodulins, transcription factors, and signaling factors, have also been identified as having functions in salt tolerance in rice. Overexpression of Ca^{2+}-dependent protein kinase OsCDPK7 or OsCDPK21 confers salt tolerance on rice [8, 155]. Ma et al. [114] reported that expression of the mouse calcineurin protein can functionally improve salt stress tolerance in rice partly by limiting Na^+ accumulation in the roots. Several studies reported that overexpression of genes encoding transcription factors such as bZIP, DREB, NAC, and zinc finger resulted in the activation of various stress-related genes and improved tolerance to salt stress [62–64, 117, 126, 174, 201, 205, 223, 228]. Even though many genes were claimed to be functionally important in salt tolerance in rice, a complete molecular scenario of salt stress response in rice remains to be established.

4 Low Temperature Stress

Rice frequently experiences low temperature stress at both seeding and reproductive stages. To understand the genetic basis of the cold stress response in rice, many QTL related to cold stress tolerance have been identified by using various mapping populations [6, 39, 93, 95, 173, 234]. One QTL controlling low-temperature germination ability, *qLTG3-1*, has been cloned by the map-based cloning strategy, and the gene was shown to encode a protein of unknown function [39, 40].

Numerous transcription factor genes have been found to play important roles in response to cold stress. The CBF/DREB (or CRT/DRE binding proteins) transcriptional regulation network plays a pivotal role in cold acclimation. Transgenic rice plants overexpressing *OsDREB1A* and *OsDREB1B* showed increased tolerance to low temperature [33, 73]. Expression of *OsDREB1F* was induced by cold stress and transgenic plants overexpressing *OsDREB1F* enhanced cold tolerance in rice and Arabidopsis [190]. The overexpression of *OsDREB1D* conferred cold tolerance in transgenic Arabidopsis plants [223].

In addition to the CBF/DREB family, several classes of transcription factors also play important roles in cold acclimation in rice. For example, transgenic plants overexpressing rice MYB transcription factors, including *OsMYB3R-2*, *Osmyb4*, and *MYBS3*, exhibited enhanced cold tolerance [27, 113, 141, 172, 185]. Overexpression of zinc finger genes such as *OsISAP1*, *OsISAP8*, and *OsCOIN* confers cold stress tolerance at the seedling stage [84, 110, 123]. Overexpression of *OsbHLH1*, *ROS-bZIP1*, *LIP19*, and *SNAC2*/*OsNAC6* also enhances cold stress tolerance of rice [21, 63, 126, 137, 167, 194]. Expression analysis revealed that eight *OsHSF* (heat shock factor) genes among the 26 members in the HSF family of rice showed induced expression in response to cold stress [121] and at least six members of the TIFY family in rice, which are jasmonic acid inducible, were strongly induced by cold [216]. These results suggest that there is a complex transcriptional regulation network for cold stress tolerance in rice.

Signaling components have also been shown to be involved in cold stress response. Protein kinases such as MAPK, CIPK, and CDPK regulate the response to cold stress in rice [196, 200, 204]. Overexpression of *OsMEK1* and *OsMAPK5* conferred tolerance to cold stresses in rice seedlings [196, 204]. Stress-responsive CIPK genes such as *OsCIPK03* also play important roles in improving the tolerance to cold stress in rice

[200]. CDPK genes *OsCDPK7* and *OsCDPK13* are positive regulators that enhance cold stress tolerance [1, 92, 155, 189].

Proteins functioning as metabolic pathways, compound synthesis, or transport have also been shown to be involved in cold tolerance in rice. For example, overexpression of many detoxification or osmotic adjustment-related genes, including ascorbate peroxidase gene *OsAPXa*, Ran GTPase gene *OsRAN2*, *CMO* (choline monooxygenase, responsible for the synthesis of glycinebetaine), and *WFT1* and *WFT2* (responsible for the synthesis of fructan) can enhance cold tolerance in rice [20, 85, 158, 168]. Other functional proteins suggested to be involved in cold stress tolerance of rice include OsSPX1 (an SPX domain containing protein), OsSFR6 (sensitive to freezing-6), OsPRP3 (a flower-specific proline-rich protein), glycine-rich RNA-binding proteins, and OsLti6a/b (hydrophobic proteins) [52, 88, 89, 122, 195, 227]. Transgenic rice plants overexpressing aquaporin genes such as *OsPIP1*;*3* and *OsPIP2*;*7* also showed enhanced cold tolerance, which is most likely due to increased water transport and maintenance of the water balance in cells [100, 103, 120, 218].

5 Other Abiotic Stresses

Sessile plants have to cope with many other abiotic stresses such as high temperature (or heat), ultraviolet (UV) light and ozone, toxic minerals. Here we briefly review the progress in genetic and genomic studies for these stresses.

5.1 Heat Stress

Heat stress response in rice is a complicated process regulated by numerous proteins in different metabolic pathways, and the genetic basis of heat tolerance in rice is generally unknown. Heat stress usually induces the synthesis of heat shock proteins (HSPs), which was universally observed in rice leaves [53, 98, 161]. A putative cold shock protein was also induced in response to high temperature in rice anther [75]. It has been reported that five elongation factors and three initiation factors for protein biosynthesis are suppressed under heat stress, and proteolysis of unfolding and misfolding proteins and the ubiquitin-mediated proteasome system are involved in the regulation of heat stress response [47].

Recently, quite a few genes were validated for their roles in heat stress tolerance in rice through the transgenic approach. Overexpression of *OsHSP17.7*, *OsHsp101*, *OsHsfA2e*, *OsHSF7*, *OsDREB2B*, *OsWRKY11*, *ZFP177*, and *OsAKR1* and knockout of *OsGSK1* resulted in enhanced heat tolerance [15, 36, 65, 91, 109, 119, 125, 181, 198, 217]. These genes encode diverse proteins including HSPs, transcription factors, glycogen synthase kinase3-like kinase, and aldo–keto reductase, implying a complex process of heat stress response in rice.

5.2 UV-Light and Ozone

Depletion of the stratospheric ozone layer leads to an increase in UV-B (280–320 nm) radiation reaching the earth's surface, and the enhanced solar UV-B radiation is predicted to reduce growth and yield of crops in the future [58]. Three QTLs associated with UV-B resistance were detected in rice, and among them, *qUVR-10* showed the largest effect by explaining about 40 % of the variation [159]. The causal gene of *qUVR-10* encodes a cyclobutane pyrimidine dimer (CPD) photolyase, and a single amino acid substitution in the CPD photolyase causes the difference in UV-B resistance between the Nipponbare and Kasalath cultivars [182]. In addition, overexpression of a rice WRKY gene, *OsWRKY89*, enhanced resistance to UV-B irradiation [191]. In another study, suppression of *OsCYP84A*, encoding a rice cytochrome P450, resulted in apparent growth retardation with obvious symptoms of damage under UV-B irradiation [157].

Tropospheric ozone is recognized as one of the most important air pollutants [3]. Frei et al. [38] identified six QTLs associated with tolerance to elevated ozone in rice. Gene expression profiling and biochemical analyses of *OzT9*

showed that enhanced antioxidants may be one of the ozone tolerance mechanisms [37]. The chromosomal segment substitution line containing the *OzT8* locus showed less reduction in photosynthetic capacity, maximum electron transport rate, and midday carbon assimilation rates under ozone stress, indicating that the *OzT8* locus confers ozone tolerance via biochemical acclimation, making it a potentially valuable target for ozone tolerance breeding [17].

5.3 Toxic Minerals

The soils of approximately 50 % of the world's arable land are acidic, and toxic levels of aluminum (Al) is the primary limitation of acid soils for rice growth [90]. Aluminum tolerance in rice is also a complex quantitative trait. So far a total of 33 Al-tolerance QTLs have been identified by using different mapping populations [115, 128–130, 197, 211, 212]. These QTLs are located on all 12 chromosomes, with four QTLs (on chromosomes 1, 2, 3, and 9) being detected in independent studies, but no causal gene has been cloned so far. On the other hand, five Al-tolerance genes have been identified in rice by using Al-sensitive mutants, *STAR1* (*Sensitive to Al rhizotoxicity 1*), *STAR2*, *ART1* (*Aluminum rhizotoxicity 1*), *Nrat1* (*Nramp aluminum transporter 1*), and *OsALS1* (homolog of Arabidopsis *ALS1*) [67, 68, 199, 213]. Recently, the genetic architecture of rice Al tolerance was investigated via biparental QTL analysis and genome-wide association analysis [34]. Forty-eight QTLs associated with Al tolerance were identified by the analysis, with three QTLs corresponding to the Al-sensitive rice mutants (*art1*, *star2*, *nrat1*).

6 Perspectives

Although hundreds of QTLs and functional genes have been identified for diverse abiotic stresses, a complete scenario of genetic or molecular bases of the tolerance of rice to single stress is still missing [203]. More integrative approaches should be adopted to reveal the genetic and molecular bases of stress resistance in rice. These approaches include, but are not limited to, genetic mapping through combination of traditional QTL mapping and association analysis; high-throughput mutant screening (especially for stress-associated genes); comparative transcriptome, proteomics, and metabolomics analyses between stress-resistant and stress-sensitive genotypes; functional characterization of more stress-associated genes; and bioinformatics or knowledge-based systematic analysis of the data from the different approaches.

Stress resistance is a synthetic and complex "trait" that can only be partially reflected by some component traits or criteria. Therefore, proper choice and accurate and efficient phenotyping of stress resistance-related traits are very important for unveiling the genetic and molecular bases of stress tolerance in rice. Phenotyping was conducted in greenhouses or tubes in most studies of gene function characterization. One needs to fit the experimental stress conditions as closely as possible to the natural stress conditions in rice. This is especially important for the translational studies of stress resistance considering the requirements of the stress resistance breeding in rice.

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Nitrogen and Phosphorus Uptake and Utilization

14

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1 Introduction

Developing nutrient efficient rice cultivars is an increasingly important objective as the continually increasing population worldwide needs to be fed from the decreasing arable land and declining natural resources. To deal with the demand, vast improvements in cultivars with higher yield potential per unit area (super rice for example) have being targeted in breeding programs. Traditionally, breeding selection is done on fully fertilized plots with a focus on potential for higher yield, making current commercial rice cultivars relatively inefficient at accessing applied fertilizers. The plants may only take up 10–15 % of applied phosphorus (P) fertilizer in the first year, with subsequent uptake rarely exceeding 50 % [17]. To obtain a higher yield, a great quantity of nitrogen (N) fertilizer has been applied during the past 20 years. The statistical data indicate that rice yield increased 26.5 % from 4,888 kg/ha in 1982 to 6,185 kg/ha in 2002, but consumption of nitrogen fertilizer increased 106.5 % in China [10]. Phosphorus is a nonrenewable resource. The exploitation of economically viable sources of rock phosphate is estimated to peak within this century [9], resulting in prohibitive prices for P fertilizer for many primary producers, especially in developing countries, increasing rice production costs. In addition, overapplication of N fertilizer often reduces rice grain yield because plants grown under excess N conditions are more susceptible to lodging and pest damage. Thus, developing crops that are less dependent on the heavy application of fertilizers is essential for the sustainability of agriculture [51].

2 Nitrogen Uptake and Utilization in Rice

Rice can be cultivated in both conventional flooded anaerobic paddy soil, where ammonium is the main inorganic N form, and upland soil, where nitrate is the dominant inorganic N form. Adaptation of the rice roots to high ammonium in the soil and low pH is associated with the regulation of plasma membrane (PM) H+-ATPase genes [54]. Rice roots have abundant aerenchyma cells, which can transfer oxygen from the shoots to roots and assist bacterial conversion of ammonium to nitrate (nitrification) at the root surface and rhizosphere [27]. Nitrification in the waterlogged paddy rhizosphere can result in 15–40 %

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of the total N taken up by rice roots, in the form of nitrate, mainly through a high-affinity transport system (HATS) [24].

3 Nitrate Uptake in Rice

Rice roots take up low concentrations of nitrate from culture medium through NAR2/NRT2, two-component nitrate transporter family members [11]. Two NAR2 and five NRT2 genes encoding HATS components have been identified in the rice genome [12, 49]. *OsNRT2.3* (locus ID R01-AP003245.4) is mRNA spliced into *OsNRT2.3a* (AK109776) and *OsNRT2.3b* (AK072215). The expression of PM-localized *OsNAR2.1*, *OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3a* is up-regulated by nitrate, light, or external sugar supply, and suppressed by ammonium, some amino acids, and high root temperature [12]. A region from position −311 to −1, relative to the translation start site in the promoter region of *OsNAR2.1*, contains the *cis*-element(s) necessary for the nitrate-dependent activation, but not light or sugar-dependent activation. However, defining a conserved *cis*-element in the promoters of the nitrate regulated OsNRT2/OsNAR2 genes is difficult, implying differential regulation pathways by nitrogen and carbon status [12].

OsNRT2.1/2.2 and OsNRT2.3a/b function at low and high nitrate concentration ranges, respectively [11, 12, 49] (Fig. 14.1). Increased OsNRT2.1 expression slightly improves seedling growth, but has no effect on N uptake [23]. OsNRT2.1, OsNRT2.2, and OsNRT2.3a all require OsNAR2.1 for nitrate transport (Fig. 14.1). Knockdown of *OsNAR2.1* by RNA interference synchronously suppresses expression of *OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3a*, impairing both high- and low-affinity nitrate transport in rice roots [11, 49]. Yeast two hybridization has shown that OsNAR2.1 interacts not only with OsNRT2.1/OsNRT2.2 but also with OsNRT2.3a. The data provide direct molecular evidence for nitrate uptake in rice and

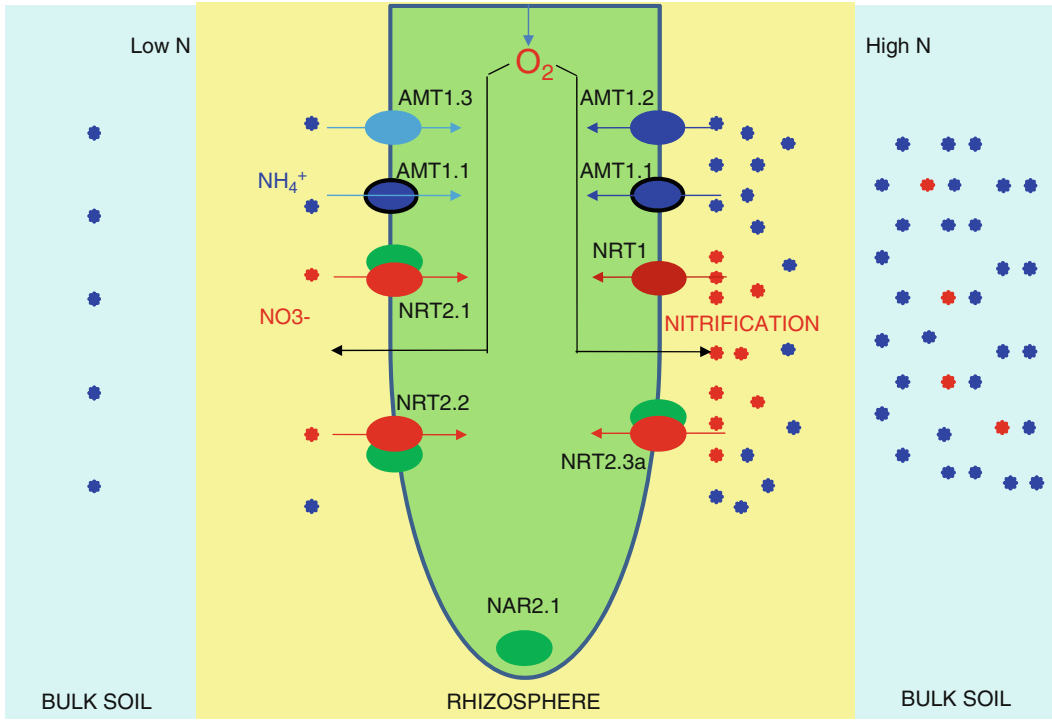

Fig. 14.1 The schematic representation of predicted functions for the rice NAR2-NRT2 two components nitrate transporters and three AMT1 ammonium transporters

demonstrate that *OsNAR2.1* plays a key role in enabling the plant to cope with variable nitrate supply [49]. OsNRT2.3b and OsNRT2.4 can function without OsNAR2.1 in nitrate transport and remobilization; the two transporters are better candidates for improving rice N utilization.

4 Ammonium Uptake in Rice

Ammonium ions are actively taken up by the roots via ammonium transporters (AMT). At least ten AMT members belonging to the AMT/MEP/Rh family have been isolated in rice [41]. OsAMT genes in rice show distinct expression profiles, i.e., constitutive expression of OsAMT1.1 and OsAMT2.1 in roots and shoots, ammonium-inducible expression of OsAMT1.2 in roots, and ammonium-inducible reversal of repressed OsAMT1.3 expression in roots [39, 43]. Glutamine (Gln), rather than ammonium, controls the expression of AMT genes in rice, indicating that the early intermediates of nitrogen metabolism, such as Gln, serve as indicators of both the environmental and cytosolic nitrogen status [41]. With respect to their transcriptional regulation, OsAMT1.1, OsAMT1.2, and OsAMT1.3 are promising candidates for mediating this adaptation (Fig. 14.1). Enhanced N uptake by over-expression of nitrate and AMT must be balanced by driving rice growth in order to avoid feedback effects on the activity of many AMT and NRT members and increased N efflux in the roots.

5 Nitrogen Assimilation and Translocation

Root-acquired nitrate is reduced to nitrite by cytosolic nitrate reductase (NR), and nitrite into ammonium by plastid nitric reductase (NiR), mainly in above-ground parts of the rice plant [26]. However, the physiological functions of NR and NiR in rice have not yet been characterized.

Ammonium acquired through AMT or from nitrate reduction is subsequently assimilated into the amide residue of Gln by Gln synthetase (GS) in the rice roots. Gln is converted by Glu synthase (GOGAT) into glutamate (Glu), a central amino acid for the synthesis of a number of other amino acids. The major forms of nitrogen in the xylem sap of rice plants are Gln and asparagine (Asn) [15]. Rice cytosolic GS1 is encoded by *OsGS1.1*, *OsGS1.2*, and *OsGS1.3* [21, 42]. The expression profile of each GS1 and the two NADH-GOGAT genes is different in terms of age and tissue specificity and response to ammonium, suggesting that each gene product has a distinct function in rice [43]. OsGS1.1 is critical for ammonium assimilation in both roots and the leaf sheath, playing a crucial role in coordinating the global metabolic network [25]. Cytosolic GS1.2 and plastidic NADH-GOGAT1 are responsible for the primary assimilation of ammonium ions in the roots. GOGATs play key roles in carbon and nitrogen metabolism, and are indispensable for efficient nitrogen assimilation in rice [32]. The chloroplast OsGS2, which is encoded by a single gene, is mainly expressed in young vegetative tissues and plays a role in N and carbon metabolism, particularly in amino acid synthesis [6].

In the top of the plant, approximately 80 % of the total nitrogen in the panicle is remobilized through the phloem from senescing organs [42]. As the major form of N in the phloem sap is Gln, GS and GOGAT are important for nitrogen remobilization and reutilization in the senescing organs and developing organs, respectively. Studies on over-expression and age- and cell type-specific expression strongly suggest that NADH-GOGAT1 is important for the reutilization of transported Gln in developing organs [43]. Expression of a barley alanine aminotransferase (AlaAT) gene in rice, driven by a rice tissue-specific promoter (OsAnt1), was shown to significantly increase the efficiency of nitrogen uptake, biomass, and grain yield in the presence of high N supply [38]. Over-expression of aspartate aminotransferases OsAAT1 and OsAAT2 in rice has been shown to result in a significant increase in leaf AAT activity and higher concentration of total amino acid and protein in the seeds [53].

6 Phosphorus Uptake and Translocation in Rice

Plant phosphate transporters (PTs) are active in the uptake of inorganic phosphate (Pi) from the soil and its translocation within the plant. Plant roots acquire Pi by active absorption via the Pi transporters into the epidermal and cortical cells of the root. In the root cortical cells, Pi must eventually be loaded into the apoplastic space of the xylem and transported to the shoot by Pi transporters. Among all known plant PTs, members belonging to the Pht1 family, which are presumed to be high-affinity PTs, have been studied more intensively [5, 33].

A database search yielded 26 potential PT family genes in rice (*Oryza sativa*). Eight conserved motifs and 5–12 transmembrane segments, most of them conserved, were identified. A total of 237 putative cis elements were found in the 2-kb upstream region of these genes. Of these putative elements, a majority were Pi-response elements and other stress-related cis regulatory elements, such as PHO-like, TATA-box-like, PHR1, and Helix–loop–helix elements, or WRKY1 and ABRE elements, suggesting gene regulation by these signals [31]. Of the 26 putative Pi transporters (carriers) identified, 13 belonged to the Pht1 family and named OsPT1 to OsPT13 (Fig. 14.2). Functional characterization of OsPT2, OsPT6, and OsPT8 has been conducted [1, 22, 30] (Table 14.1). The high-affinity Pi transporter OsPT6 is broadly involved in Pi uptake and translocation in plants. However, OsPT2, unlike other Pht1 members, is a low-affinity Pi transporter that might function primarily in the Pi translocation process [1]. OsPT8 is a high-affinity Pi transporter and plays crucial role in Pi and arsenate (As) uptake and transportation [22, 48]. Over-expression of *OsPT2* and *OsPT8* results in excessive Pi accumulation in shoots leading to Pi toxicity (leaf necrosis and inhibited plant growth) under conditions of high Pi supply [22, 31].

Because enhanced expression of Pi transporters increases Pi uptake and transportation, we expect that plants over-expressing PTs will have improved growth performance under conditions of low Pi supply. However, this expectation has not been observed in solution or soil experiments [30, 52] (Fig. 14.3). These results imply that constitutive ectopic expression of both low- and high-affinity Pi transporters physiologically impairs the nutrient balance required for normal metabolism in plants, or the higher expression of PTs consumes more energy and has a higher metabolic cost.

Using tissue-specific promoters to enhance the expression of PTs, such as root hair-specific promoters, may be an alternative strategy for utilizing PTs to improve the Pi uptake ability of plants. On the other hand, the coordinated regulation of PT expression may be required to improve P uptake and transportation ability.

7 Pi-Signaling Pathway and Homeostasis Regulation in Rice

Maintenance of Pi homeostasis in plants is essential for plant growth and development, and is achieved by coordinated acquisition of Pi from the soil, translocation of Pi from roots to shoots, and remobilization of internal Pi [34]. Two PHR gene products have been isolated from rice (OsPHR1 and OsPHR2) based on amino acid sequence homology with AtPHR1, a central Pi-signaling regulator [37] (Table 14.1). Transgenic plants have shown that both genes are involved in the Pi-signaling pathway; over-expression of OsPHR2 mimics Pi-starvation stress with enhanced root elongation and proliferated root hairs, resulting in an excessive accumulation of Pi in shoots under Pi-sufficient conditions. OsPHR2 regulates proliferation of root hair growth and root elongation, suggesting that OsPHR2 is involved in both systematic and local Pi signaling and plays multiple roles [52]. The Pi-signaling pathway downstream of OsPHR2 is similar to that of AtPHR1, whereby miR399 (a PHR1 target) reciprocally regulates *PHO2* at the posttranscriptional level [3, 8, 14, 28]. miR399-mediated PHO2 cleavage is controlled by IPS1, a noncoding RNA, through target mimicry [13]. PHO2 functions as an

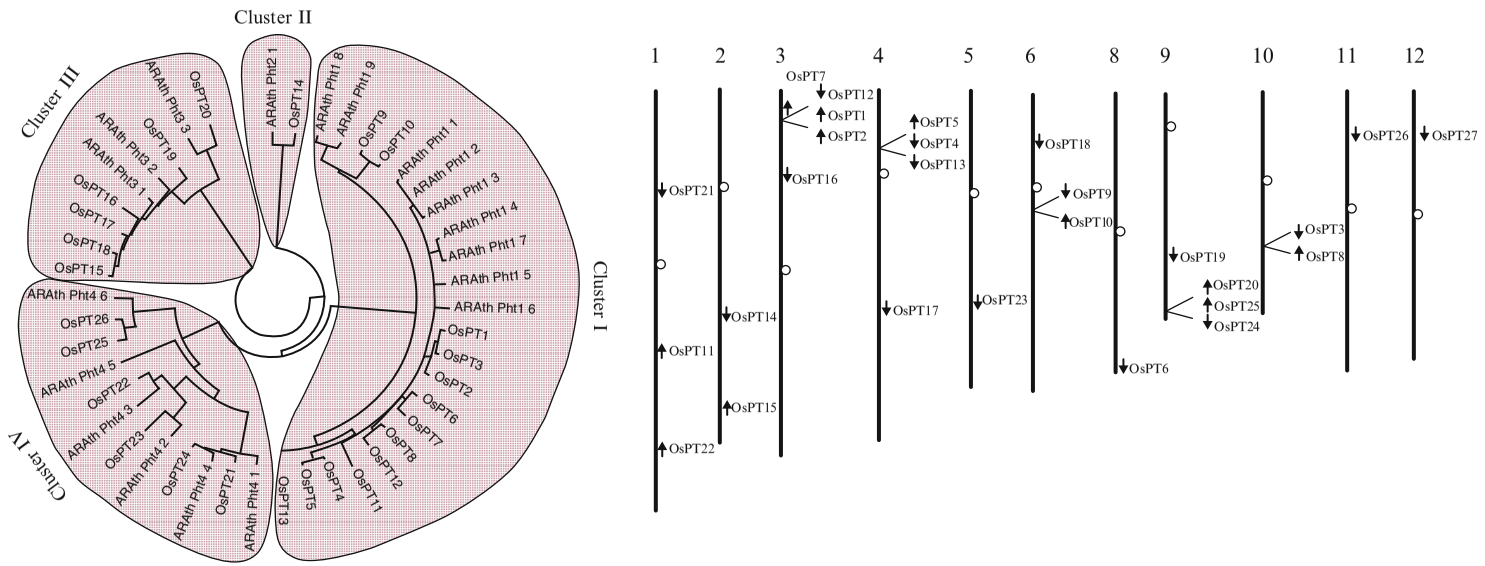

Fig. 14.2 Phylogenetic relationship between PT family genes in rice (OsPT) and Arabidopsis. The joint unrooted tree was generated using MEGA4 with the neighbor-joining method. Bootstrap values from 1,000 replicates are indicated at each branch (*left*). Distribution of OsPT Genes on Rice Chromosomes. *White ovals* on the chromosomes (*vertical bar*) indicate the position of centromeres. The *arrows* next to the gene names show the direction of transcription. Chromosome numbers are indicated at the top of each bar (*right*)

Table 14.1 Abbreviations of genes with roles in Pi uptake and translocation described in text

Gene locus	Full gene name	Protein function	References
OsPHR2 (LOC_Os07g25710)	PHOSPHATE STARVATION RESPONSE2	MYB transcription factor	[53]
OsPHO2 (LOC_Os05g48390)	PHOSPHATE2	Ubiquitin E2 conjugase (UBC24)	[19, 45, 47]
OsPT2 (LOC_Os03g05640)	PHOSPHATE TRANSPORTER PHT1;2	Pi translocation	[1]
OsPT6 (LOC_Os08g45000)	PHOSPHATE TRANSPORTER PHT1;6	Pi uptake and translocation	[1]
OsPT8 (LOC_Os10g30790)	PHOSPHATE TRANSPORTER PHT1;8	Pi uptake and translocation	[22]
OsIPS1 (AY568759)	INDUCED BY PHOSPHATE STARVATION1	Nonprotein coding RNA	[18]
OsIPS2 (AK240849)	INDUCED BY PHOSPHATE STARVATION2	Nonprotein coding RNA	[18]
OsmiR399	microRNA 399		[30]
OsmiR827 (LOC_Os02g39610)	microRNA 827		[29]
OsPTF1 (LOC_Os06g09370)	PHOSPHATE TRANSCRIPTION FACTOR	bHLH transcription factor	[51]
OsSPX1 (LOC_Os06g40120)	SPX (SYG1/Pho81/XPR1) DOMAIN1	SPX domain protein	[30, 45]
OsSPX3 (LOC_Os10g25310)	SPX (SYG1/Pho81/XPR1) DOMAIN2	SPX domain protein	[47]
OsPHF1 (LOC_07g09000)	PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1	WD40-repeat	[7]

ubiquitin-conjugating E2 enzyme (UBC24), and loss of function of PHO2 leads to an accumulation of excess Pi in the shoots [2, 3] and up-regulates transcription of *OsIPS1* and *OsSPX1* in rice roots [30]. In rice and barley, two P starvation-inducible, noncoding RNA (OsIPS1/2 and HvIPS1/2) have been identified [18, 20] (Table 14.1). The expression of two low-affinity Pi transporter genes, HvPHT1.6 and HvPHT1.3, is positively correlated with that of HvIPS1, but not HvIPS2, suggesting that HvIPS1 plays a distinct role in the regulation of low affinity Pi transporters and P remobilization.

Although transgenic rice plants over-expressing *OsPHR2* (PHR2(Ov)) and *pho2* mutant exhibit accumulation of excess Pi in the shoots [44, 52], the Pi transporters that play important roles in Pi uptake and transportation under the regulation of OsPHR2 and OsPHO2 may be different. This conclusion is supported by the observation that the low-affinity Pi transporter, OsPT2, contributes to the accumulation of excess Pi in shoots, which occurs in *OsPHR2*(*Ov*) plants but not in *pho2* mutants [1, 30]. OsPHR2 positively regulates *OsPT2* by physical interaction with an upstream regulation of OsPHO2 in roots [30]. Additional evidence of the differential regulation of OsPT2 by OsPHR2 and OsPHO2 is provided by the observation that OsSPX1, which is encoded by a gene specifically responsive to Pi signaling, is a negative regulator of OsPHR2 but not OsPHO2 [30, 46] (Fig. 14.3). The response of plants to Pi signaling and maintenance of Pi homeostasis in cells is clearly defined by a complex feedback loop.

8 Posttranscriptional Regulation of Pi Transporters

A multiple-level regulation model of PTs has been suggested from important findings in Arabidopsis that the PHT1 family encoding the high-affinity PTs require PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR (PHF1) to exit the endoplasmic reticulum (ER) and reach the PM to transport Pi [16]. PHF1 acts on PHT1 upstream of vesicle coat protein COPII formation, and additional regulatory events occur during PHT1 trafficking to determine its exit from the ER and PM stability, which is regulated by the Pi concentration in the medium [4]. Phosphoproteomic and mutagenic analyses indicate Ser-514 phosphorylation status modulating

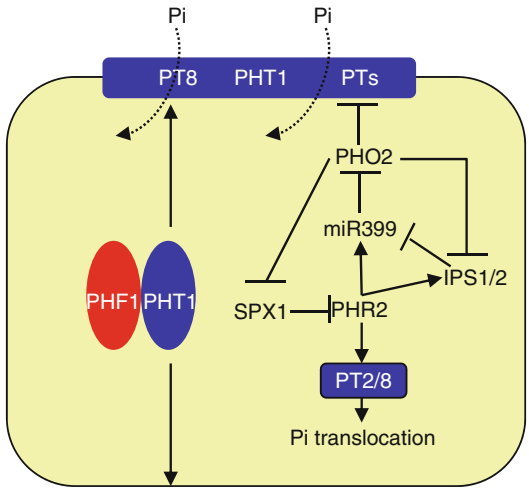

Fig. 14.3 Model of the feedback network regulating Pi-uptake and translocation defined by OsPHR2, OsSPX1, and OsPHO2 in roots of rice. Internal Pi status regulates the activity of MYB transcription factor PHOSPHATE STARVATION RESPONSE 2 (OsPHR2) by unknown mechanisms. OsPHR2 activates a circuit of microRNAs (miR399) and noncoding RNAs (IPS1/2), which regulates OsPHO2, a member of the E2 ubiquitin conjugase family (UBC24), expression at the level of PHO2 mRNA degradation. Over-expression of OsPHR2 and repression of PHO2 causes upregulation of transcript levels of genes encoding high-affinity Pi/H+symporters of the PHOSPHATE TRANSPORTER 1 (PHT1) family in roots, including low-affinity Pi transporter OsPT2 [1], high-affinity Pi transporter OsPT8 [22], and OsPT9 and OsPT10 which showed dual affinity (Wang et al. personal communication), followed by accumulation of Pi in shoots [30, 44, 46, 52]. In addition of the reciprocal regulation, OsPHR2 can physically upregulates OsPT2. Repression of OsPT2 can dramatically reduce the accumulation of Pi in shoots driven by overexpression of OsPHR2 but not reduce the accumulation of Pi in shoots in OsPHO2 mutant. OsPSX1 acts as repressor of OsPHR2 but not of OsPHO2. OsPHO2 negatively regulates OsSPX1 and OsIPS1/2 at transcript levels [30]. At post-transcription level, OsPHF1 acts as PHT1-specific chaperone to facilitate PHT1 transit through the ER to reach to plasma membrane [7]

PHT1.1 ER export. Based on current knowledge, the model of PHT1 regulation involves transcriptional control of the quantitative and spatial distribution of PHT1 in plant tissues in response to environmental modifications. One step in post-translational regulation requires PHF1 to act as a PHT1-specific chaperone to facilitate PHT1 transit through the ER. A second posttranslational regulation step concerns phosphorylation of particular Ser residues located in the C-terminal end of PHT1. A third regulatory step is the down-regulation of PHT1 concentration at the PM through Pi level-dependent endocytosis, followed by routing to lytic vacuoles for degradation. Most, if not all, posttranslational regulatory events identified in Arabidopsis are conserved [4]. Isolation and functional analysis of OsPHF1, the homolog of PHF1 in Arabidopsis, supports this hypothesis [7] (Table 14.1).

Screening for rice mutants defective in Pi uptake by taking advantage of the increased arsenate resistance phenotype identified several rice mutants with *phf1* alleles. Phosphate uptake and translocation were severely reduced in these mutants. The data indicate that mutation of *OsPHF1* results in retention of the low-affinity Pi transporter OsPT2 and high-affinity Pi transporter OsPT8 at the ER (Fig. 14.3). The function of PHF1 required to facilitate PHT1 transit through the ER that is similar between Arabidopsis and rice provides an example of expectations that can be deduced from sequence comparisons to extrapolate nutrient efficiency from Arabidopsis to crops.

Protein alignment analysis also indicates conserved phosphorylation events for Ser 514 at the C-terminal end of PHT1 in Arabidopsis and rice. The interesting question is how PHF1, a PHT1-specific chaperone, modulates PHT1 to prevent phosphorylation. Elucidation of the biochemical mechanism underlying the modulation of PHT1 by PHF1 is required to increase the efficiency of PHT1 through molecular manipulation at post-transcriptional levels.

9 Genotypic Variation in Critical P Levels

Genotypic variation exists for phosphorus efficiency (PE) in most crops, including rice [47], suggesting that genetic factors or the genomic background could be exploited in breeding programs for P-efficient rice cultivars.

Two components have been thought to contribute to genotypic PE; namely, the internal P use efficiency (PUE = grams of biomass produced

per milligram P taken up) and P uptake ability (PUA = total P taken up per plant). The P acquisition efficiency (PAE = milligrams of P taken up per unit root size) has also been suggested to indicate the PUA [45]. To maximize the PE of a crop system, genotypes should be selected for low grain P (LGP) to minimize P removal [35]. Thus, the ultimate P-efficient genotype would have high PAE and PUE traits in combination with an LGP trait. Using 30 genotypes Rose et al. [36] observed a genotypic variation in total plant P uptake (0.14–0.59 mg/plant) and shoot PUE (1.45–3.61 g shoot dry weight/mg), with a tight negative association between them ($R^2 = 0.70$), which clearly indicates that PUE is strongly affected by genotypic differences in P uptake, and high PUE (low shoot P concentrations) is most likely the result of P starvation due to insufficient P uptake. The lack of a significant relationship between shoot PUE and biomass in the plus-P treatment suggests that biomass is driven (or limited) by factors other than P nutrition, and other than PUE in the plus-P treatment [36].

10 Prospect

The response of plants to Pi signaling and the maintenance of Pi homeostasis in cells are clearly defined by a complex feedback loop. Many pieces of the Pi regulatory network are still missing, which requires future work aimed at solving the mystery of how plants sense Pi, transmit signals locally and at long distance, and maintain nutrient homeostasis to utilize Pi efficiently in coordination with other nutrients.

The present findings provide the potential for us to improve crops with nutrient uptake efficiency. Increased transcripts of several Pi and nitrate transporters and their regulators, such as OsPHF1 and OsNAR2.1, endow rice plants with higher ability to absorb Pi and nitrate, and tolerance to low Pi and nitrogen stresses. The knowledge on the efficient physiological utilization of the absorbed nutrients in plants is a key challenge to utilize these findings to improve crops with higher yield under economic fertilizer application levels.

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Yield

15

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1 Introduction

The world's population continues to increase rapidly and is expected to grow from about seven billion today to 9.3 billion by 2050. Given this rise, food shortage is becoming an even more serious global problem. To meet the demand for food, improvement of yield is essential and is the most important target in crop breeding.

Along with maize (*Zea mays*) and wheat (*Triticum aestivum*), rice (*Oryza sativa* L.) is one of the world's three major cereal crops, providing a staple food for more than half of the world's population. In the middle of the twentieth century, when Asia had a similar food shortage problem, the International Rice Research Institute (IRRI) developed IR8, a high-yielding, semi-dwarf rice variety known as miracle rice. IR8 was bred by crossing a good-tasting Indonesian variety, Peta, and a native Taiwanese semi-dwarf variety, Dee-geo-woo-gen, carrying the *semi-dwarf 1* (*sd1*) gene [1]. IR8 dramatically increased rice production, and as a result, the feared food crisis was averted in Asia during the 1960s and 1970s. This remarkable achievement was referred to as a part of the green revolution. However, for overcoming food shortages, a second green revolution is required [2], and this need has led to attempts to breed high-yielding and ground-breaking cultivars in various areas.

The yield of rice can be divided into five major components: number of plants per unit area, tiller number per plant, grain number per panicle, grain size, and grain fertility. The branching habit of shoots and panicles has attracted attention as a significant trait in rice grain production, because it is closely linked with yield potential via tillering of the vegetative shoot and rachis branching of the panicle.

The most crucial agronomic traits (e.g., yield, field disease resistance, and food quality) are generally governed by many genes known as QTLs. Draft genomic sequences of two rice subspecies, *O. sativa* L. ssp. *japonica* cv. Nipponbare and *O. sativa* L. ssp. *indica* 93-11, were reported in 2002 [3, 4], and subsequently the International Rice Genome Sequencing Project completed the sequencing of the entire rice genome of Nipponbare in 2005 [5, 6]. This achievement has allowed researchers to perform detailed genetic analysis, such as QTL analysis, using DNA-based marker linkage maps. Since then, many studies have identified and characterized QTLs and genes important for agronomic traits [7–9]. In this chapter, we focus on recent advances in identification and characterization of major QTLs associated with high crop yield such as grain number, grain size and filling, and tillering.

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2 Panicle Branching Pattern

The panicle is one of the most important organs affecting grain yield, because its development directly regulates seed grain number as a factor dictating sink size. The rice panicle consists of a main axis and primary, secondary, and higher-order rachis branches (Fig. 15.1). Seed grains in the panicle are attached at the end of each rachis branch; therefore, the grain number is assumed to nearly equal the number of terminal branches. Therefore, to reveal the mechanism conferring high yield, it is important to elucidate how development of the rachis and branching structure is controlled genetically. However, almost all studies of panicle traits have targeted traits controlling number, not length. Studies of length traits have focused mainly on panicle and primary rachis length, rarely on the relationship between the number of branches and the length of each order branch. Therefore, how panicle length is controlled genetically remains unknown.

The panicle is controlled by various traits, and measuring these traits across many samples tends to require a lot of time and labor. To obtain data automatically, we developed image analysis software: PAnicle STructure Analyzer for Rice (PASTAR) and the PASTA Viewer [10]. Using this method, we analyzed some rice cultivars that produce an exceptionally large number of grains and performed QTL analysis for 18 panicle traits, including length traits, in a segregating F_2 population derived from a cross between a leading *japonica* variety, Koshihikari, and a cultivar with an exceptionally large number of grains, NP-6, which is classified as a New Plant Type [11]. As a result, three regions in which more than four QTLs were concentrated as a cluster were found (Fig. 15.2). The relationship between each branch's length and number was detected as both independently and dependently controlling branching pattern. These results suggest that different genetic mechanisms could control the length and number of rachis branches in rice panicles. When genetic control of each panicle component is identified,

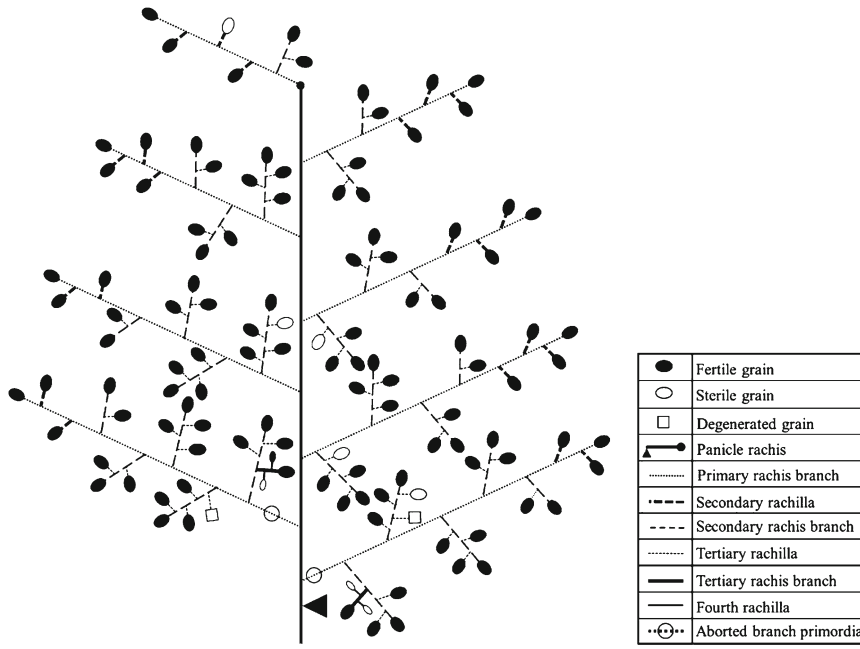

Fig. 15.1 Illustration of rice panicle structure (alteration from Ikeda et al. [10])

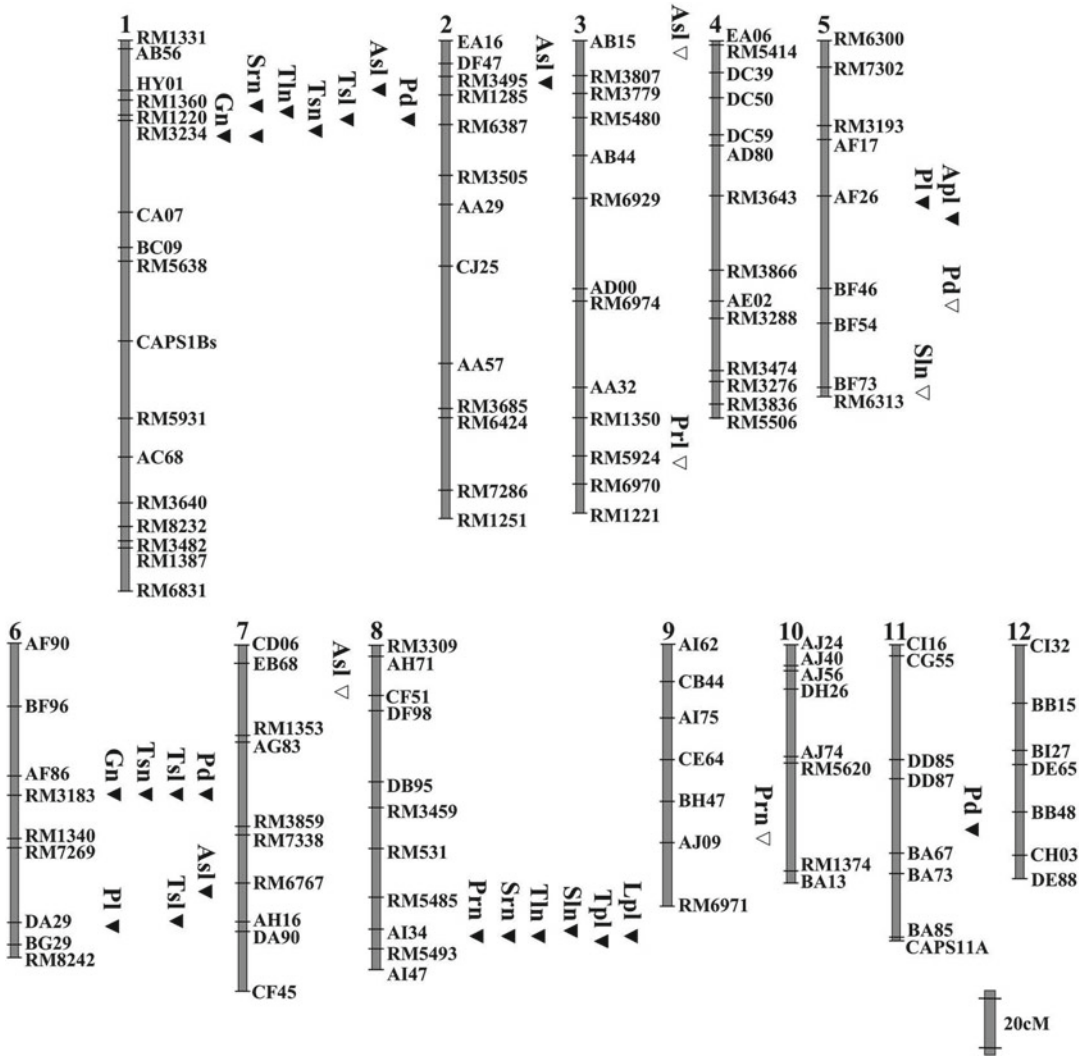

Fig. 15.2 Linkage map showing the location of QTLs for 14 panicle traits in the Koshihikari/NP-6 F_2 population based on results of QTL analysis performed with Windows QTL cartographer using phenotype data automatically obtained by PASTAR and PASTA Viewer. QTL peaks with a significant logarithm threshold of the odds score ($P<0.05$) are shown by *black arrowheads* (enhanced by the NP-6 allele) and *outlined arrowheads* (Koshihikari allele). *Gn* grain number, *Prn* primary rachis branch number, *Srn* secondary rachis branch number, *Tln* total number of lateral branches on the primary rachis branch, *Tsn* total number of lateral branches on the secondary rachis branch, *Sln* total number of secondary rachis branches on the longest primary rachis branch, *Pl* panicle length, *Prl* panicle rachis length, *Tpl* total length of primary rachis branch, *Apl* average length of primary rachis branch, *Tsl* total length of secondary rachis branch, *Asl* average length of secondary rachis branch, *Lpl* longest primary rachis branch length, *Pd* panicle density (alteration from Ikeda et al. [10])

it will be possible to design panicle branching patterns for different purposes, for example, one pattern that has a long panicle with many primary rachis branches and another that has a long primary rachis with many lateral branches on the primary rachis branch.

3 Grain Number

Several QTL analyses for rice yield traits have been performed [12–17]. In 2005, *grain number 1a* (*Gn1a*) was detected and identified as the

most effective QTL increasing grain number through QTL analysis using backcrossed inbred lines between the *indica* cultivar Habataki and the *japonica* cultivar Koshihikari [18]. *Gn1a* encodes a rice cytokinin oxidase/dehydrogenase, OsCKX2, and its reduced expression in inflorescence meristems induces cytokinin accumulation, which leads to an increase in the number of flowers. A nearly isogenic line of *Gn1a* had many grains about 45 % more than those of Koshihikari, and thus an effect of *Gn1a* on rice yield was indicated. Therefore, this was the first report that identified a useful gene for an agronomic trait in rice.

Natural variation in *grain number*, *plant height, and heading date 7* (*Ghd7*) was also defined using QTL analysis from both $F_{2:3}$ and recombinant inbred line populations derived from a cross between *indica* cultivars Zhenshan 97 and Minghui 63 [19, 20]. *Ghd7*, encoding a CCT (CO, CO-LIKE, TIMING OF CAB1) domain protein, increases grain number, plant height, and heading date and acts upstream of *Early heading date 1* (*EHD1*) and *Heading date 3a* (*Hd3a*) in the photoperiod flowering pathway [21]. *Ghd7* controls the heading date through its enhanced expression under long-day conditions to repress the expression of *Hd3a*, likely through *Ehd1*, thus delaying flowering and increasing plant height and panicle size. Similarly to *Ghd7*, *Ghd8*/*Days to heading 8* (*DTH8*), which encodes the rice Hook Associated Protein 3 (OsHAP3) subunit of a CCAAT-box binding protein, also pleiotropically regulates grain yield, heading date, and plant height [22, 23]. Through regulating the expression of *Ehd1* and *Hd3a*, *Ghd8*/*DTH8* delays flowering time under long-day conditions, whereas it promotes it under short days. *Ghd8* also increases the number of tillers and primary and secondary branches through positively regulating a key gene controlling tillering and branching, *MONOCULM 1* (*MOC1*) (see below).

Dense and erect panicle 1 (*dep1*) was also identified as a natural variant of the *DEP1* locus that increases grain number and leads to formation of dense, erect panicles based on analysis of an F_2 population between *japonica* and *indica* varieties (Q169×93-11 and W101×NJ6) [24]. *DEP1* encodes a previously unknown protein with a phosphatidylethanolamine-binding-like domain. *DEP1* can accelerate the cell division of rice and lead to higher grain number.

Additionally, *WEALTHY FARMER'S PANICLE* (*WFP*) and *Ideal Plant Architecture 1* (*IPA1*) were independently identified and encode the same protein, rice SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 14 (OsSPL14), an SBP-box protein that is the target of miRNA156 [25, 26]. *WFP* from *indica* variety ST-12 and *IPA1* from Shaoniejing increased the grain number because of a significantly higher number of primary branches per panicle. *OsSPL14* mRNA, which contains a target sequence of microRNA, OsmiR156, is highly expressed in the shoot apex and primordia of primary and secondary branches; it reduces tiller number and promotes panicle branching.

Recently, we identified a major QTL *STRONG CULM 2* (*SCM2*) using Sasanishiki-Habataki chromosome segment substitution lines [27]. Positional cloning of *SCM2* revealed that it is identical to *ABERRANT PANICLE ORGANIZATION 1* (*APO1*), encoding an F-box-containing protein orthologous to *Arabidopsis thaliana UNUSUAL FLORAL ORGAN* (*UFO*), involved in controlling the rachis branch number of the panicle [28]. A nearly isogenic line of *SCM2* plants in the Koshihikari background showed enhanced culm strength and pleiotropic phenotypes, such as increased branch and grain number per panicle. These pleiotropic effects of *SCM2* should be very useful for raising crop productivity of rice.

On the other hand, map-based cloning of a mutant of *LARGER PANICLE* (*LP*) revealed that it encodes a Kelch repeat-containing F-box protein [29]. Morphological analyses revealed that mutant panicles produce more inflorescence branches, in particular primary branches, and contain more grains with increased 1,000-grain weight. Compared to wild-type plants, the total grain yield per mutant plant is also increased, indicating that mutants might be used as a gene resource in rice breeding for higher grain yield.

4 Grain Size and Filling

In breeding applications, grain size is usually evaluated by grain weight, which is positively correlated with several characters including grain length, grain width, and grain thickness. Grain size is also a highly important quality trait in rice, because increased grain size usually reduces grain quality.

Some QTLs for grain weight have been detected. *GS3*, identified in progeny derived from a cross between Minghui 63 (large grain) and Chuan 7 (small grain), is a major QTL for grain length and weight [30]. The GS3 protein is composed of four putative domains: a plant-specific organ size regulation (OSR) domain at the N-terminus, a transmembrane domain, a tumor necrosis factor receptor/nerve growth factor receptor (TNFR/NGFR) family cysteine-rich domain, and a von Willebrand factor type C (VWFC) domain at the C-terminus, and such overall structure is similar to that of DEP1 protein [24]. The OSR domain is both necessary and sufficient for *GS3* to function as a negative regulator of grain size; on the other hand, the TNFR/NGFR and VWFC domains show an inhibitory effect on OSR function, whereas loss-of-function mutations of these domains produced very short grains [31]. However, the biological function of GS3 in controlling grain length and weight is not clear.

A QTL influencing grain width and weight, *GW2*, was identified using an F_2 population between a *japonica* variety WY3 (large grain) and a high-quality elite *indica* variety Fengaizhan-1 (small grain) [32]. *GW2* encodes a RING-type E3 ubiquitin ligase. A nearly isogenic line containing a loss-of-function allele (NIL-GW2) showed increased cell numbers, resulting in a larger (wider) spikelet hull, and faster grain filling, resulting in enhanced grain width, weight, and yield. These results suggest that *GW2* negatively regulates cell division by targeting its substrates for ubiquitin-dependent degradation by the 26S proteasome.

Shomura et al. and Weng et al. reported cloning a domestication-related gene, *qSW5*/*GW5*, a QTL involved in rice grain width [33, 34]. The QTL was observed in an F_2 population between the *japonica* variety Nipponbare and the *indica* variety Kasalath. Recently, *GS5* was found to control rice grain size by regulating grain width, filling, and weight using a double haploid population derived from a cross between the *indica* variety Zhenshan97 and H94 [35]. *GS5* encodes a putative serine carboxypeptidase and functions as a positive regulator of grain size.

The rice *GRAIN INCOMPLETE FILLING 1* (*GIF1*) gene (also referred to as rice *CELL-WALL INVERTASE 2* (*OsCIN2*)) encodes a cell-wall invertase that hydrolyzes sucrose to glucose and fructose and is involved in carbon partitioning during early grain filling. The higher expression of the wild-type *GIF1* allele leads to smaller grains compared with the cultivated *GIF1* allele [36]. Based on population genetic analysis, Wang et al. reported that *GIF1* and another cell-wall invertase gene, *OsCIN1*, have experienced strong domestication selection, with the target of selection in the *GIF1* gene being the promoter region and that in *OsCIN1* the coding region [37].

5 Tillering

Plant architecture is determined through shoot development and panicle formation, and the increase in number of branches leads to maximum yield potential. Therefore, crop production has focused on tillering as a vitally important trait.

MOC1 was predicted to be a master regulator of rice tillering, involved in axillary meristem initiation and tiller bud outgrowth [38]. This gene encodes a GRAS family nuclear protein highly homologous to the tomato *Lateral suppressor* (*LS*). Loss of function of this gene in tomato leads to a branchless phenotype due to failure of axillary meristem initiation [39]. *moc1* mutants almost completely lose tillering ability and produce only one main culm. Therefore, these results suggest that both *MOC1* and *LS* positively regulate establishment of axil identity.

Among the mutations in rice affecting tillering, the rice *Teosinte Branched 1* gene (*OsTB1*) is a counterpart of maize *TB1*. *TB1* is a negative regulator of lateral branching and contributed to the domestication of maize from its ancestor

teosinte [40, 41]. Both *OsTB1* and *TB1* encode a TCP-domain transcription factor, and the *fine culm 1* (*fc1*) mutant has a loss-of-function allele of *OsTB1* [42]. Recently, Minakuchi et al. suggested that *FC1* integrates the signaling pathways of both strigolactones and cytokinins derived from auxin, which leads to regulating the outgrowth of axillary buds [43].

The process of tiller formation in rice might be similar to the branching mechanism of *Arabidopsis*, and these plants have been proposed to share some regulatory components. In recent studies, some genes homologous between rice and *Arabidopsis* have been reported, for example, rice *MOC1* and *Arabidopsis LATERAL SUPPRESSOR* (*LAS*) [38, 44], rice *DWARF 3* (*D3*) and *Arabidopsis MORE AXILLARY GROWTH 2* (*MAX2*) [45], rice *DWARF 17* (*D17*)/*HIGH TILLERING DWARF 1* (*HTD1*) and *Arabidopsis MAX3* [46], and rice *DWARF 10* (*D10*) and *Arabidopsis MAX4* [47]. Rice *DWARF* genes and *Arabidopsis MAX* genes are connected in their respective regulation of tiller elongation and apical dominance; therefore, these findings suggest that the same mechanism operates in monocot and dicot plants.

6 Conclusion

Since the emergence of next-generation sequencing technologies [48–51], it is now possible to obtain whole-genome scale information at a highly accelerated rate. This advance in sequencing technology has led to new opportunities to explore global genomic and transcriptomic landscapes. Consequently, genome-wide analyses are relying on the use of high-throughput sequencers such as Roche's 454 Genome Sequencer FLX, Illumina's Solexa Genome Analyzer, and Applied Biosystems SOLiD System [52–57]. Several QTLs for yield traits have been identified (Table 15.1); however, exploration of genes regulating important agronomic traits is expected to

Table 15.1 List of QTLs/genes controlling yield traits in rice

Traits	QTL/gene name	Encoded product	References
Grain number	*Gn1a*	Cytokinin oxidase/dehydrogenase	[18]
Grain number	*Ghd7*	CCT domain protein	[19–21]
Grain number	*Ghd8*/*DTH8*	OsHAP3 subunit of a CCAAT-box binding protein	[22, 23]
Grain number	*DEP1*	PEBP-like domain protein	[24]
Grain number	*WFP*	OsSPL14	[25]
Grain number	*IPA1*	OsSPL14	[26]
Grain number	*SCM2*	F-box-containing protein	[27]
Grain number	*APO1*	F-box protein	[28]
Grain number	*LP*	Kelch repeat-containing F-box protein	[29]
Grain length and weight	*GS3*	Transmembrane protein	[30, 31]
Grain width and weight	*GW2*	RING-type E3 ubiquitin ligase	[32]
Grain width	*qSW5*/*GW5*	Novel nuclear protein	[33, 34]
Grain width, filling, and weight	*GS5*	Serine carboxypeptidase	[35]
Grain filling	*GIF1*	Cell-wall invertase	[36, 37]
Tillering	*MOC1*	GRAS family nuclear protein	[38]
Tillering	*OsTB1*/*FC1*	TCP-domain transcription factor	[42, 43]
Tillering	*D3*	F-box leucine-rich repeat protein	[45]
Tillering	*D17*/*HTD1*	Carotenoid-cleaving dioxygenase (CCD)	[46]
Tillering	*D10*	CCD8	[47]

advance ever more rapidly. Combining marker-aided gene and QTL pyramiding, tailor-made breeding, which enables the design of new cultivars integrating the genes needed for breeding desirable traits, makes it possible to realize these advances more effectively. Additionally, with this approach, every breeding program will be able to expand according to local or regional preferences and needs and can be applied to breeding of other cereal crops.

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Grain Quality

16

Yanchun Yu, Rod A. Wing, and Jiayang Li

1 Introduction

Rice is a major human food crop that feeds over 50 % of the world's population. During the past decades applications of the "green revolution" and hybrid rice technologies have rapidly improved rice productivity. However, the previous breeding goal in last century was over shed light on yield; accordingly, the rice quality was not paid more attention to and the grain quality was not increased as fast as yield. The rice qualities include the appearance, milling quality, nutritional quality, and eating and cooking quality (ECQ) [1]; appearance and ECQs have received more attention than other qualities in rice from consumers. Given that the rice endosperm is the major eating part and starch is the predominant component in dehulled grains (composed of 76.7–78.4 % in polished rice), the rice ECQs are thus thought to be mainly influenced by starch properties [2]. The improvement in rice grain quality has been increasingly demanded by consumers and has become a priority for rice breeders and geneticists [3–5]. Studies on the elucidation of the molecular mechanisms underlying appearance and ECQs of rice have made significant advances recently. This chapter will review current progress in understanding the genetics and molecular biology of rice grain quality, focusing on ECQs and appearance of rice grains.

2 ECQs of Rice Grains

2.1 Properties Affecting Rice Grain ECQs

Three physicochemical properties of starch have been considered as determinants of rice grain ECQs: amylose content (AC), gel consistency (GC), and gelatinization temperature (GT) [6, 7].

Amylase content (AC), also called AAC (apparent amylose content) because of the measurement method used to assay amylose content by iodine staining, can detect both amylose and long chain amylopectin [8]. Based on AC values, rice varieties can be divided into two classes: glutinous rice (extremely low AC, 1–2 %) and non-glutinous rice (AC >2 %). Non-glutinous rice can be further classified into four types: very low AC (2–10 %), low AC (10–20 %), medium AC (20–25 %), and high

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AC (>25 %). In general, *japonica* rice contains low to very low AC values and tends to be sticky, moist, and tender when cooked. Generally, *japonica* rice is highly preferred in northern-east Asia, including China, Japan, and Korea. In contrast, *indica* rice contains high AC and cooks soft and fluffy in texture and is favored in southern and southeastern Asian regions [9].

Gel consistency (GC), as a good index of cold paste-viscosity of cooked rice, refers to the gel running distance of digested grains by KOH in a flattened tube [10]. Based on gel length, rice varieties can be divided into three classes: hard (<40 mm), medium (40–60 mm), and soft (>60 mm). Cooked rice with hard GC tends to be dry and fluffy after cooling, whereas soft GC rice remains moist and continues to remain soft after cooling. In general, soft GC rice is more desirable by consumers [11].

Gelatinization temperature (GT) is the temperature at which starch granules start to lose crystallinity and birefringence by irreversible expansion that alters the starch surface from polarized to a soluble state [12]. To measure rice grain GT, several approaches have been developed, such as differential scanning calorimetry (DSC), rapid visco analyser (RVA), and alkali spreading value (ASV). Based on the ability of seed resistant to alkali digestion, varieties can be classified into three categories: high GT (>74 °C), intermediate GT (70–74 °C), and low GT (<70 °C) [12]. Given that resistance of seeds to alkali digestion is antagonistic with GT, high GT varieties are substantially hard to be digested when eaten, and vice versa.

In general, rice varieties with fine ECQs can be characterized as having medium AC (15–17 %), soft GC (>60 mm), and low GT (<70 °C) [13].

2.2 Genetic Molecular Studies of AC

2.2.1 Inheritance of AC

Studies on domestic varieties have shown that long grain types (*indica* rice) can be characterized by relatively high amylose content (24–28 %), whereas typical short and medium grain varieties (*japonica* rice) have relatively low amylose content (15–20 %) [14], which has accelerated the application of AC as a selection criterion in rice breeding programs [14]. At present, three inheritance models of AC have been proposed. According to the first model, high AC is controlled by a single dominant major gene, along with some minor genes and/or modifiers [15, 16]. The second model supports the existence of two dominant or complementary genes that control the AC trait [17]. The third model hypothesizes that AC is a quantitative trait controlled by multiple genes [18]. In general, the first model is more popularly accepted by geneticists and breeders [15, 16, 19–22] and is supported by the transgressive segregation in F_2 populations derived from low AC and intermediate AC parents [9, 16].

2.2.2 QTLs for AC

With the development of high-density marker linkage maps in rice, a series of studies for QTL analysis of rice grain quality have been conducted. Table 16.1 summarizes QTL that have been identified in rice for AC over the past decades. A doubled-haploid (DH) population, derived from the anther culture of an *indica*/*japonica* hybrid, was first utilized to identify QTL for AC [1] which identified two QTL on chromosomes 5 and 6. The major QTL on chromosome 6 explained 91.1 % of the variance and was closely linked with the previously identified *Wx* gene, which had been shown to control AC in both maize and rice [23, 24]. Thereafter, several groups have detected the *Wx* locus [6, 25–31] as the major AC QTL, as well as other minor effecter loci in diverse populations [1, 26, 28, 32–34]. Besides the popular DH population [6, 29–31], other populations, like F_2, BC_2, BC_3, and CSSL, have also been developed and applied for positioning of AC loci [25, 27, 33, 34]. In addition, two pairs of epistatic QTL involving QTL-by-environment interactions (QEs) of AC have been detected as well [6]. These data indicate that the regulation of AC is complex and associated with a number of heritable factors and environmental conditions.

2.2.3 Genes Regulating Amylase Content

Since the identification of two classical maize mutants defective in amylase in kernels [23] and endosperm in the last century [35], mutants with (Beijing) a similar waxy phenotype have been

Table 16.1 Identified QTL for amylose content in rice

Cross	Population type	QTLs	Chr.	Marker interval	LOD value	Additive effect	Variance explained (R2)	Reference
ZYQ8/JX17	DH	qAC-5	5	RG573-C624	2.67	−3.32	11.8	[1]
		qAC-6	6	Wx	28.39	−8.52	91.1	
Zhenshan97/Minghui63	F2&RIL	Wx	6	RM170-RM190	69	2596.2^{MS}	N.A	[25]
IR64/Azucena	DH	AAC	7	RG375/RG477	2.61	0.76	6	[32]
IR64 x Oryza rufipogon	BC2F2	AC	6	RM170	14.63	−0.88	21.9	[27]
V20A/IRGC 103544	BC3(TC)F1	qAC-6	6	RG653	4.2	0.06	8	[33]
		qAC-12	12	RG574	6.4	0.07	8	
Caiapo/IRGC103544	BC3F1/DH	qAC-3	3	RM7-RM251	3.7	−2.73	21.5	[28]
		qAC-6	6	RM190-RM253	19.3	−2.60	73.7	
		qAC-8	8	RM230-RM264	3.1	−1.85	10.9	
Asomonori/IR24	CSSL	qAC-8	8	G1149-R727	3.7	1	17.1	[34]
		qAC-9a	9	XNpb36-XNpb103	2.5	1	13.4	
		qAC-9b	9	C609-C506	2.4	1.1	19.2	
		qAC-12	12	XNpb189-2-XNpb24-2	2.5	1.4	8	
Wuyunjing2/Zhenshan97B	DH	qAC-6	6	RM190-RM510	35.5	−9.4	61.8	[29]
Nanjing11 × Balilla	DH	qAC-6	6	S10372-Wx	31	4.8	72.8	[31]
PSB Rc10/Nip	DH	qAC6a	6	RM469-RM170	38	5.88	65	[30]
		qAC6b	6	RM170-RM190	62.33	6.37	74	
		qAC6c	6	RM197-RM225	20.91	4.77	63	
Zhenshan97/H94	DH	qAC6A	6	RM190-RM587	65.8	5.83	54.87	[6]
			6	Cgene-MRG5119	6.2	0.83	1.1	
			11	RM209-RM229	10.8	−1.07	1.85	
			12	RM270-RM235	7.9	−0.73	0.85	
KDML105/CT9993	RIL	qAC3	3	RM81-C155	N.A	1.86	11.28	[26]
		Qac4	4	G177A-GA2-7	N.A	0.63	15.99	
		qAC6	6	Waxy-RM204	N.A	4.48	58.69	
		qAC7	7	OSR22-RM10	N.A	0.96	9.18	

Note: MS = MS effect

subsequently identified in rice, barley, wheat, potato, sorghum, and amaranths [24, 36–41]. The maize *Wx* gene was cloned in 1983 [42] and used subsequently as a probe to identify the homologous gene in rice [43], resulting in the identification of a 2.4-kb transcript that has been fully characterized [22, 44]. Surprisingly, besides the normal 2.4-kb transcript, an extra aberrant 4.0-kb transcript has also been found in glutinous rice from cultivar PI291667. Given that the translation of the maize and barley *Wx* genes begins in exon 2, raising the possibility that the extra-long *Wx* sequence may be attributed to the retention of the first intron [41, 45]. Sequencing the aberrant fragment of the *Wx* transcript from rice cultivar Hanfeng revealed that the entire intron 1 was indeed present in the aberrant long cDNA, and that the 3′ end of *Wx* cDNA included proper termination features of Poly(A) and an AATAAT sequence. In addition, it shares exactly the same sequence as its counterpart in non-glutinous rice [24, 44]. Furthermore, Northern-blot and Western-blot analyses of multiple varieties showed that low AC cultivars accumulate substantial amounts of un-spliced long *Wx* transcripts, including intron 1, and that high AC cultivars are depleted of the un-spliced transcript [24]. All of these data support the 5′ end retention hypothesis. Actually, previous data have shown that two Wx proteins, Wx^a and Wx^b, were present in rice. Wx^a is characteristic of *indica* rice with high AC and Wx^b is mainly found in *japonica* rice with intermediate AC [37]. In contrast to the Wx^a-type rice, a T to G change in the 5′ splice site of intron 1 was detected in Wx^b and a significant reduction of Wx^a transcript amount was also observed [46]. These data further prove that the role of the G to T change in the first intron of the *Wx* gene affects transcription levels and final amylose biosynthesis. In the rice *du1* mutant, Wx^b transcription [47] and protein accumulation were reduced significantly [48]. Therefore, *Du1* may participate in both *Wx* transcriptional and translational regulation. A recent study revealed that *Du1* encodes a Prp1 protein, a component of spliceosome. The defect of Prp1 in the *du1* mutant leads to a specific decrease of the splicing efficiency of *Wx* rather than other starch biosynthesis-related genes (SSRGs) [49]. Furthermore, the AC level of a *du1*/*wx* double mutant is almost the same as that of *Du1*/*wx* mutant, which is much lower than that of the *du1*/wx^b mutant. In mammals, the U5-102kD Prp1 protein interacts with U4/U6 snRNPs and bridges the two particles through its TPR elements [50]. However, the molecular basis of why a *Du1* mutant specifically affects the splicing of the Wx^b pre-mRNA, and how Du1 recognizes the first intron of the *Wx* transcript remains to be elucidated [49]. In addition, the MYC (for v-myc avian myelocytomatosis viral oncogene homolog) protein has been shown to interact with EREBP (for ethylene responsive element binding protein) and bind to the *Wx* gene promoter, which results in enhanced transcription of the *Wx* gene [51, 52]. All these results show that transcriptional and posttranscriptional regulation of the *Wx* gene is crucial for the expression of *Wx* and amylose biosynthesis.

2.3 Genetic Molecular Studies of Gel Consistency (GC)

2.3.1 Inheritance of GC

Gel consistency (GC) is a good index of cooked rice texture for cold paste-viscosity, especially among rice varieties with high amylose content and varieties with hard, medium, and soft GC levels have been selected for by rice breeders with their breeding goals. To explore the genetic inheritance of the three types of GC levels, bulked F_2 and F_3 seeds were analyzed and the hard GC was found to be controlled by a single dominant gene [19]. Subsequent investigations using a single grain analysis resulted in a similar conclusion [11, 53]. The inheritance of GC was further explored by utilizing various populations, such as F_2, B_1F_1, and B_2F_1, derived from parents with hard and soft, hard and medium, and medium and soft GC properties as well, and a major gene with multiple alleles was identified [54]. These studies all suggested that the hard GC is controlled by a single locus/gene. However, analyses using a 6X6 diallel set excluding reciprocals and involving contrasting parents revealed the predominance

of additive gene action in the regulation of GC trait expression [55]. A similar result was also observed by Yi and Chen [56], implying that the GC is unlikely controlled by a major gene.

2.3.2 QTL for GC

Like AC, a number of studies have been conducted to understand the genetic basis of GC [25–29, 31–34, 57, 58]. Because of the negative correlation between AC and GC, it was suggested that GC is controlled by *Wx*, or another gene closely linked to the *Wx* locus [5, 25]. To identify QTL responsible for GC, an RIL population was developed and a single QTL with a large MS effect was detected on chromosome 6, which corresponded well with the *Wx* gene [25]. Furthermore, using two additional RIL populations, major QTL for GC on chromosome 6 had been detected as well, accounting for 57 % and 53 % of the phenotypic variation, respectively [26, 57]. The *Wx* locus responsible for GC was also identified with different DH populations [5, 29, 31]. All of these results indicate that the *Wx* locus is the major candidate gene controlling GC. Besides *Wx*, several minor effect QTL (Table 16.2), located on different chromosomes, were also identified from different populations [25–29, 31–34, 57, 58]. Therein, two loci identified using a DH population from similar GC parents were found not to overlap with known SSRG genes [32]; therefore, it should be interesting to clone these novel GC regulatory genes.

2.3.3 Genes Regulating GC

Based on genetic studies, numerous reports suggested that *Wx* was the primary determinant of GC; however, no direct molecular evidence was available until recently. The major QTL on chromosome 6, qGC-6, was represented with a DH population and this locus was finally characterized by using chromosome segment substitution lines [5]. *qGC-6* encodes a granule-bound starch synthase (Wx), which has been well-documented for its role in AC. A comprehensive comparison revealed several polymorphic sites, including a previously known G/T transition between CJ06/TN1 parents. Although the complementation experiment had confirmed the role of the *Wx* gene in GC, which or how many SNP or InDel sites in the *Wx* gene are pivotal for GC and starch biosynthesis remains to be determined. Besides *Wx*, other starch-related genes, like *ALK* and *SBE3*, have been shown to play a role in regulating GC as well. When a functional *ALK* gene from low GC rice (Shuangkezao, *indica* type) was transferred into the GC intermediate Nipponbare (*japonica* type), transformants showed a decreased GC value [59]. However, the opposite phenotype was also observed in rice plants over-expressing the *ALK* gene, in which the GC value increased significantly [7]. Therefore, it will be interesting to elucidate the molecular mechanism of *ALK* in regulating GC in future. In addition, transformation of a functional *SBE3* gene into *japonica* cv. WYJ7 decreased the GC value significantly [7], indicating that *SBE* may function as a minor gene contributing to GC.

2.4 Molecular Genetic Studies of Gelatinization Temperature

2.4.1 Inheritance of GT

The inheritance of GT has been studied extensively, but its mode has not been found to be consistent, not only in the number of genes responsible for GT but also in the nature of dominance-recessive relationships [60, 61]. Puri et al. reported the segregation patterns of GT in five reciprocal cross populations derived from three different GT (high, medium, and low) parents, but and could not identify consistent mode of inheritance, suggesting the lack of a major gene in controlling GT [61]. A similar conclusion was also drawn from studies on crosses between the cultivar 9192 and the mutant *mahsuri* [60] and an *indica* cytoplasmic male sterile (CMS) line and its restorer lines, respectively [62]. However, a bimodal frequency distribution was detected in an F_2 population between SD7 and 72-3764, indicating that a major locus is governing GT [17]. Moreover, a few additive genes with major effects, along with modifier genes, were proposed as well [17]. Lastly, it should be pointed out that GT inheritance has been shown to be affected by

Table 16.2 Identified QTL for gel consistency in rice

Cross	Population type	QTLs	Chr.	Marker interval	LOD value	Additive effect	Variance explained (R2)%	Reference
Zhenshan97/Minghui63	F2:3 & RIL	qGC-6	6	c952-C1496	N.A	10722.7$^{(MS)}$	N.A	[25]
KDML105/CT9993	RIL	qGC6-1	6	Waxy-RM225	N.A	16.98	53.1	[26]
		qGC6-2	6	RG64-R2171	N.A	5.18	10.5	
		qGC7-1	7	RG375/RG477	N.A	8.20	12.77	
IR64/Oryza rufipogon	BC2F2	qGC	6	RM50	14.63	3.57	6.6	[27]
IR64/Azucena	DH	qGC-1	1	RG331/RG810	2.71	–3.7	9	[32]
		qGC-7	7	RG477/PGMSO.7	3.54	–4.65	13	
Asomonori/IR24	CSSL	qGC-4	4	C445-Ky4	4.9	1	–3.8	[34]
		qGC-6	6	XNpb209-C688	2.3	1	–3.3	
		qGC-11	11	XNpb257-C1350	8.3	1.1	–9.3	
Wuyunjing2/zhenshan97B	DH	qGC-1	1	RM294B-RM306	2.3	7.8	5.7	[29]
		qGC-2	2	RM190-RM510	2.5	7.9	6.3	
		qGC-6	6		33.7	22.4	59.7	
Zhenshan97/H94	DH	GC6a	6	RM170-RM589	7.3	–6.87	10.88	[6]
		GC6b	6	RM190-RM587	29.6	–11.87	32.52	
		GC6c	6	C gene-MRG5119	5.9	–2.08	1	
		Epistatic	1	RM543-RM302	N.A	2.5	1.45	
Nanjing11/Balilla	DH	qGC6-1	6	Wx-R1952	16.3	–12.9	47	[31]
		qGC6-2	6	RM508-RM435	6.2	–8.4	19.7	
PSB Rc10/Nip	DH	qGC2	2	RM71-RM2634	3.2	–4.58	4	[30]
		qGC6a	6	RM469-RM170	14.48	–11.44	23	
		qGC6b	6	RM170-RM190	23.81	–14.15	35	
		qGC6c	6	RM197-RM225	10.61	–11.32	23	
		qGC8	8	RM350-RM342A	3.59	–4.8	4	
Zhenshan97/Minghui63	RIL	qGC-1-1	1	C904-R2632	N.A	5.02	N.A	[57]
		qGC-6-2	6	C952-Waxy	N.A	17.2	53	
TN1/CJ06	DH	qGC-2	2	RM3732-RM492	2.96	–16.76	17.8	[5]
		qGC-3	3	RM514-RM85	3.05	–18.40	14.6	
		qGC-6	6	RM540-RM587	12.17	–32.30	52.4	

Note: MS = MS effect

environment, whereby high air temperature after flowering raises GT and lower temperatures have the opposite effect [60, 63].

2.4.2 QTLs for GT

As mentioned above, the inheritance model of GT had been well explored using crosses among diverse cultivars. To identify the genes responsible for GT, a number of QTL mapping populations (e.g., F_2, RILs, BILs, DH, and CSSLs) have been developed and are listed in Table 16.3. Among them, DH populations have been widely employed [1, 6, 29, 30, 32] and resulted in the identification of important two QTL [1]. One QTL was found to be a major contributor to GT and was delimited to the CT506-C235 interval on chromosome 6. This QTL was recognized by several labs using diverse population types [6, 26, 28, 29, 58]. The CT506-C235 region contains a known locus, *ALK*, which is responsible for alkali digestion, indicating the pivotal role of *ALK* for GT. Besides *ALK*, the *Wx* gene, with both major and minor effects, has been identified as well [6, 26, 28, 29, 32, 34].

2.4.3 Cloning ALK—A Gene That Regulates GT

To clone ALK, a locus that has been shown to regulate GT, segregating F_2 populations were utilized to map and fine map *ALK* to a 9-kb region on chromosome 6 between the genetic markers R2147 and C1478 [12, 64]. BlastX analysis revealed a partial ORF that encoded a soluble starch synthase IIa (SSIIa or ALK) within the 9-kb region and three amino acid substitutions in the ALK genes of the parental lines C Bao (low GT) and Shuangkezao (high GT) were detected [12]. Considering the previous data that ALK is functional in elongation of medium chain-length amylopectin [65], it is therefore interesting to understand the role of different amino acid substitutions in ALK in regulating chain-length elongation. To do that, an elaborate set of expression shuffle constructs with diverse amino acid residue substitutions in the wild-type cv IR36 ALK sequence were established, and the purified proteins were utilized to test their SSIIa activity in vitro [66]. Results showed that the replacement of Val-737 with Met-737 abolished SSIIa activity in chain-length elongation from the degree of polymerization (DP) <12 to DP 13–25, indicating a critical role of the Val-737 site for ALK function. In contrast, substitution of the site Phe-781 only partially affected ALK activity, and double mutations of residues Gly-604 and Phe-781 were shown to enhance the deficiency of ALK activity. These observations suggest that the Phe-781 is an important secondary site and that Gly-604 may interact positively with Phe-781 to determine ALK activity [66].

2.5 Complex Network Regulating Starch Biosynthesis

As rice grain ECQs are triploid endosperm traits, their inheritance patterns are very complicated because the genetic expression of an endosperm trait in cereal seeds is conditioned not only by the triploid endosperm genotype, but also by the diploid maternal genotype, and additional cytoplasmic components [1, 67, 68]. Several studies concerning the inheritance of rice grain ECQs have been conducted over several decades, but the data are not always consistent. The generally accepted model is that nuclear gene expression is the predominant mechanism affecting rice grain quality, though a few studies have suggested that the chloroplast genome may play a role as well [56, 69].

Based on current knowledge, starch structure is determined by four classes of enzymes: ADP glucose pyrophosphorylase (AGPase), starch synthase (SS), starch branching enzyme (BE), and debranching enzyme (DBE) [70, 71]. As previously described, the two SS enzymes, Wx and ALK, have been well documented to affect AC and GT. Accumulating evidences have shown that Wx is involved in amylose biosynthesis, especially in the formation of extra-long chain fractions [72, 73]. In contrast, ALK has been shown to determine the elongation of short- to medium-length starch chains (DP 13–25) [12, 59, 66]. In addition to Wx and ALK, other SSRGs, such as OsSS1 [74], OsSSIIIa [75], isoamylase1 [71], branching enzyme [76, 77], and pullulanase [78], confer their unique or overlapping roles on

Table 16.3 Identified QTL for gelatinization temperature in rice

Cross	Population type	QTLs	Chr.	Marker interval	LOD value	Additive effect	Variance explained (R2)	Reference
ZYQ8/JX17	DH	qASS-6	6	CT201-RZ450	6.19	1.23	24.6	[1]
		ALK	6	CT506-C235	27.04	2.33	82.4	
Zhenshan97/Minghui63	F2&RIL	Wx	6	C952-C1496	N.A	N.A	N.A	[25]
KDML105/CT9993	RIL	qGT2	2	RG73-RM6	N.A	0.24	12.22	[26]
		qGT6a	6	C1478-RZ667	N.A	2.21	60.30	
		qGT6b	6	RM3-RM238	N.A	0.68	8.57	
IR64/Azucena	DH	qGT	6	Amy2A/RG433	2.44	−0.35	10	[32]
Caiapo/IRGC103544	BC3F1/DH	qGT6-1	6	RM190-RM253	32.5	0.87	50.1	[28]
		qGT6-2	6	RM253-RM162	10	−0.87	44	
Asomonori/IR24	CSSL	qGT-3	3	C1677-R3156	5.1	−1.9	20.5	[34]
Wuyunjing2/zhenshan97B	DH	qGT-6	6	RM276-RM121	34	3.52	80.3	[29]
Zhenshan97/H94	DH	ASV1	1	RM297-RM128	11.8	0.35	3.63	[6]
		ASV6a	6	RM190-RM587	27.1	0.7	14.22	
		ASV6b	6	RM111-RM253	31.3	−0.83	19.58	
		ASV6c	6	RM190-RM587	46.2	−1.15	38.12	
PSB Rc10/Nip	DH	qGT2	2	RM3294-RM6233	3.48	0.19	3	[30]
		qGT6a	6	RM469-RM170	10.95	0.35	9	
		qGT6b	6	RM170-RM190	19.39	0.47	16	
		qGT6c	6	RM197-RM225	3.43	0.23	4	
		qGT6d	6	RM7023-RM3330	52.52	−0.93	62	
		ASV11	11	RM202-RM484	7.9	−0.33	3.82	

Table 16.4 Characterized starch biosynthesis and regulation-related genes in rice

Classification	Gene name	Other name	Effects in fine structure of starch	Physiochemical change in mutant	References
SS	GBSSI	Wx	Amylose decreased	Decreased AC	[73]
	SSI	N.A	Decreased in DP 8-12 amylopectin; Increased in DP 6-7 and 16-19dp amylopectin	N.A	[74, 80]
	SSII-3	ALK	Amylopectin DP13-25 decreased	Decreased GT	[59, 66, 80]
	SSIIIa	N.A	DP 6-9 and DP 16-19 amylopectin decreased; DP 10-15 and DP 20-25 increased	Decreased GT	[75, 79, 80]
SBE	Sbe1	N.A	Decrease in DP >37 and DP 12-21, increased in DP <10, and slight increase in DP 24-34 amylopectin	Decreased GT	[77]
	SbeIIb	Amylose extender(ae)	Decreased in DP 8-12 amylopectin	Increased GT	[76]
DBE	Isoamylose	ISA or sugary 1	Increased in DP8-12; depleted in DP 13-23 amylopectin	Decreased GT	[71]
	Pullulanase	Pull	Increased short chains of DP ≤12	Slightly decreased GT	[83]
TF	RSR1	N.A	Increased in DP 5-8 and 18-38	Increased AC	[51]
			DP 9-17 decreased of amylopectin	Decreased GT	
	DU1	N.A	Amylose decreased	Decreased AC	[49]

the formation of fine starch structure and rice quality regulation as well (Table 16.4).

As the major SS isozyme in developing endosperm, *SS1* mutations have not been shown to have an influence on the size or shape of grains and starch granules, or on the crystallinity of endosperm starch. However, *ss1* mutants do lead to an obvious decrease in DP 8–12 chains and an increase in DP 6–7 and DP 16–19 amylopectin chains [74]. In contrast, the *ssIIIa* mutant exhibited obvious deficiency phenotypes in grain shape, and the internal amylopectin chains, DP 6–9 and DP 16–19, were shown to decrease, while DP 10–15 and DP 20–25 chains increased [75, 79]. The opposite chain-length deficiency in *ss1* and *ssIIIa* mutants strongly indicates their distinct and overlapping functions in amylopectin biosynthesis. While the double *ss1 ssIIIa* null mutant is sterile, double mutants with a leaky *ss1* and a null *ssIIIa* allele are fertile. These results further support the role of both SSI and SSIIIa in starch biosynthesis in rice endosperm and seed development [80].

SBE1 and SBEIIb are two well-characterized starch branching enzymes from rice. The mature grain of *sbe1* mutant looks like that of the wild type, not only in appearance but also in the size and weight, whereas *sbeIIb* mutant has significantly smaller kernels with a floury appearance [77]. Biochemical analysis revealed that the AC level of endosperm starch in *sbe1* mutant was similar to that of the wild type, while significant decreases in both long chains (DP >37) and short chains (DP12-21) of amylopectin were observed [77]. In contrast, the *sbeIIb* mutant was specifically reduced in short DP <17 chains, with the greatest decrease in DP 8–12 chains to alter the structure of amylopectin in the endosperm [76].

For starch DBEs, two genes, *ISA1* [71] and *PULL* [78, 81], have been well characterized. An antisense transgenic line of *isa1* contains increased levels of short chains (DP <12) and is depleted in intermediate-size chains (DP 13–23) [71, 82]. In contrast, the *pull* mutant showed an increased level of short chains of DP <13 [78, 81, 83]. The *pull*-null/mild *isa1* double mutant still retained starch in the outer layer of the endosperm tissue, while amounts of short chain amylopectin (DP ≤7) were higher than that of the *isa1* mutant. These data indicate that the function

of PULL is partially overlapping with that of ISA1 and its deficiency has less impact on the synthesis of amylopectin than that of ISA1 [83].

Even though QTL mapping and cloning of starch synthesis genes have provided useful information for rice grain quality, it has been difficult to isolate QTL/genes with minor effects in order to elucidate the complex network of starch synthesis due to limited germplasms used in single experiments. To gain a broader understanding of the starch synthesis and its regulatory network, 18 SSRG genes were selected as candidates to carry out an association study [7]. With this approach, a fine network of rice starch biosynthesis and regulation was established. As a result 10 of the 18 SSRGs have been shown to be associated with rice grain quality. Both *Wx* and *ALK* are two central determining factors affecting all three properties (AC, GC, and GT). *Wx* functions as the sole major gene for both AC and GC, but as a minor gene affecting GT, consistent with QTL mapping results, whereas ALK was found to be the sole determinant for GT, but as a minor gene affecting AC and GC. In addition, several genes were shown to be associated with minor effect on starch biosynthesis: *SSIII-2*, *AGPlar*, *PUL,* and *SS1* for AC; *AGPiso*, *ISA,* and *SBE3* for GC; and *SSIV-2*, *ISA,* and *SBE3* for GT [7]. So far, this is the first genome-wide study of how the allelic diversity of SSRGs has collectively been shown to regulate rice grain quality via the starch biosynthesis network.

3 Grain Appearance

3.1 Features Affecting Rice Grain Appearance

The quality of grain appearance is mostly determined by grain shape as specified by grain length (GL), grain width (GW), grain length/width ratio (GS), and grain chalkiness [84]. Although preferences for rice grain appearance vary by consumers, long and slender rice is generally preferred by most of consumers in north-America and Asian countries [84]. Based on the Chinese national criteria for rice quality, the grain length of grade I rice is 6.5–7.5 mm for *indica* and 5.0–5.5 mm for *japonica* rice; and the ratio of grain length/width is >3.0 for *indica* and 1.5–2.0 for *japonica* rice.

3.2 Inheritance of Grain Shape

Grain shape is determined by grain length, grain width, and/or the ratio of grain length to width. Studies on grain shape have been explored extensively [17, 85–87], not only because of its elegant appearance from a visual sense, but also because of its strong effect on yield improvement due to its positive correlation with grain weight [88]. Genetic studies from different crosses among *japonica* X *japonica* and *indica* X *japonica* have showed that there are no obvious differences in reciprocal backcross progeny and that continuous distribution patterns were observed in progeny populations, suggesting that grain length is governed by quantitative maternal nucleic genes [89–91]. Similarly, observations using F_2 populations derived from crosses between varieties with different grain width also detected a continuous grain width distribution, suggesting a polygene model for grain width [17, 86, 92–94]. However, numbers of genes responsible for grain shape appear to be variable and are probably dependent on their genetic backgrounds. For example, Liu [95] found that the grain length segregation ratio in a specific F_2 population was 3:1 suggestive of a single gene model controlling grain shape, whereas Xu et al. found a transgressive segregation which correlated with a QTL gene model that controlled grain shape in a similar F_2 population [90].

3.3 QTLs for Grain Shape

Over 20 QTL mapping studies have been conducted to understand the genetics of grain shape and hundreds of responsible loci had been detected: 119 for grain length, 90 for grain width, and 60 for grain length/width ratio [96]. Among these studies, some QTL have been shown to account for major effects (Table 16.5) [1, 84,

97–103]. Lin et al. utilized F_2 populations derived from two pairs of *indica* parents with significant differences in grain shape to detect QTLs affecting grain length, width, and thickness. Consequently, 14 QTLs were detected [99], 5 for grain length, 2 major and 2 minor genes for grain width, and 1 major and 4 minor genes for grain thickness. Huang et al. developed a DH population from IR64 and *Azucena* parents for QTL mapping. Twelve QTL affecting grain shape were localized onto 5 different chromosomes, among them 4 for grain length, 5 for grain width, and 3 for length/width ratio [104]. Using an F_2 population, Redona and Mackill [100] identified 7 QTL for GL, of which two loci on chromosomes 3 and 7 with high LOD values had already been identified by Takeda and Saito [105] and Takamure and Kinoshita [106]. It should be pointed out that the QTL on chromosome 7 for GL also affected GW and grain length/width ratios as well. Xing et al. [107] utilized an RIL population to analyze QTL for grain shape and identified a major QTL (GW5) on chromosome 5 which is responsible for all three features of grain shape indicating that these loci may function as positive regulators to increase grain weight. By using $F_{2:3}$ and RIL populations derived from crosses between Zhenshan97 and Minghui63, a major QTL for GL on chromosome 3 (GS3) and a major QTL for grain width on chromosome 5 (GW5) were identified [84]. GS3 was also detected by several groups using different mapping populations [28, 84, 104, 108–110], indicating its general role in grain length determination. In addition, several independent studies identified a number of QTL for rice grain width using diverse mapping populations [96]. Among them, WG5 and WG7, which were first described by Lin using an F_2 population derived from Tesanai 2×CB1128 [99], were found to have significant contributions to the total grain width [84, 101, 103]. Recently, by using an F_2 population derived from Zhonghua11×Baodali (a variety with larger grain size), two major QTL for GW located on chromosomes 3 and 6 were identified [97]. In addition, a major QTL on chromosome 8 (GW8) has been detected by several groups in a number of populations [111]. These QTL have laid a solid foundation for further gene cloning and understanding of the regulation of grain width.

3.4 Genes Affecting Rice Grain Shape

Because of the strong correlation between grain shape and yield, significant efforts have been made to fine map and clone genes that regulate grain shape [110–113]. To clone the major QTL for grain length, *GW3.1* or *GS3* [84, 102, 114] was first fine-mapped to a 93.8-kb interval on chromosome 3 using a BC_2F_2 population derived from a cross between *Jefferson* and *O. rufipogon*. GS3 was then cloned using an F_2 population derived from Minghui63 and Chuan7 as was proposed to be a loss-of-function mutation of a putative transmembrane protein [113]. Protein domain analysis indicated that GS3 may have four putative domains: a plant-specific organ size regulation (OSR) domain at the N terminus, a transmembrane domain, a tumor necrosis factor receptor family cysteine-rich domain, and a von Willebrand factor type C (VWFC) domain at the c-terminus. To elucidate the roles of these domains, a series of transformation assays with different protein truncations were conducted, showing that the OSR domain is essential and sufficient for GS3 to function as a negative grain size regulator [112].

A number of studies have been reported on the mapping of GW QTL (Table 16.5). The *GW2* gene was first cloned by using a BC_3F_2 population derived from a cross between WY3 and Fengaizhan [115]. *GW2* encodes a RING-type E3 ubiquitin ligase and WY3 GW2, truncated by 310 amino acids, still possesses intrinsic E3 ligase activity, suggesting that the C-terminal of GW2 is not essential for substrate degradation. Mutations of *GW2* result in increased cell numbers and acceleration of grain milk filling rate, which in turn enhances grain width, and yield [115]. Shomura et al. [101] performed a QTL analysis with an F_2 population derived from Nipponbare×Kasalath and identified and subsequently cloned *qSW5* (*GW5*), which explained 38 % of the natural variation in the F_2 population. Sequence compar-

Table 16.5 Major QTL identified for grain shape in rice

Cross	Population type	QTLs	Chr.	Marker interval	LOD value	Additive effect	Variance explained (R2)%	Reference
Tesanai/CB1128	F_2	wg5	5	RG9-RG182	6.19	−0.14	19.7	[99]
		wg7	7	RG650-RG4	11	−0.17	32.5	
Labelle/BlackGora	F_2	GL-3	3	RZ452-RZ284	9.95	−0.29	20.9	[100]
		GL-7	7	RG711-RG650	8	−0.23	17.2	
		GB-7	7	RG711-RG650	10.54	0.13	22	
		GS-3	3	RZ403-RZ452	10.19	−0.18	21.4	
		GS-7	7	RG711-RG650	12.89	−0.08	26.2	
Zhenshan97/Minghui63	$F_{2:3}$	GL3	3	RG393/C1087	41	−0.57	63.8	[84]
		GW5	5	RG360-C734a	20.6	0.18	55.2	
Zhenshan97/Minghui63	RIL	GL3	6	RG393/C1087	33.8	−0.88	57.6	
		GW5	12	RG360-C734a	16.5	0.31	44	
Jefferson/O.rufipogon	BC_2	Gw3.1	3	RZ672-RZ474	6.49	N.A	10.9	[109]
		GW3.2	3	RM130-RG1356	6.69	N.A	11.3	
Nipponbare/Kashlath	NIL	Tgw6	6	C358	4.12	N.A	18.7	[98]
Asominori/IR24	CSSL	qGL-1	1	R210-C955	5.3	−0.15	18.2	[103]
		qGL-3	3	R19-C1677	5.9	0.29	32.8	
		qGW-5	5	R3166-R569	5.4	−0.16	27	
Asominori/IR24	CSSL	qGL-3a	3	C80-C1677	27.79	−0.26	32.2	[102]
		qGL-7	7	XNpb379-XNpb268	15.97	−0.2	19.1	
		qGL-9	9	XNpb339-C796C	12.16	0.17	10.7	
Nipponbare/Kashlath	F_2	qSW-5	5	C263-R413	N.A	N.A	38.5	[101]
Baodali/Zhonghua11	F_2	GW3	3	RM282-RM6080	N.A	N.A	N.A	[97]
		GW6	6	RM6836-RM1340	N.A	N.A	N.A	

Wg weight of grain, *GL* grain length, *GB* grain breadth, *GW* grain width, *SW* seed width

ison of *qSW5* between the two parents revealed a 1,212-bp deletion in (Nipponbare) and several SNPs. Further complementation experiments and sequencing of *qSW5* from additional cultivars revealed that the 1,212-bp deletion played an important historical role in rice domestication [101]. To understand the grain width difference between Asominori and IR24, Weng et al. [116] also found the same 1,212-bp deletion in the *GW5* gene [116]. Recently, two previously identified GW QTLs, GS5 and GW8, were fine-mapped and cloned [84, 107, 117, 118]. GS5, which encodes a putative serine carboxypeptidase belonging to the peptidase S10 family and has a PF00450 consensus domain, may function as a positive regulator of grain size by affecting grain width, filling, and weight [117]. In addition, sequencing the promoters of 51 rice accessions from diverse geographic regions identified three haplotypes that appear to be associated with grain width [117].

Previous studies revealed a major QTL *GW8.1* for grain width [119], which was fine-mapped to a 306-kb region [111] on chromosome 8. However, there is no report to date on the cloning of this gene. Recently, Wang et al. [118] reported the cloning of a major gene (*GW8*) on chromosome 8, which does not appear to be allelic to *GW8.1*. GW8 encodes a Squamosa promoter-binding protein-like 16, which belongs to the SBP domain family of transcription factors and shares homology with TGA1, a domestication syndrome gene associated with the formation of naked grains in maize.

4 Perspective for Rice Quality Improvement

Development of new cultivars with improved grain quality for eating, cooking, and grain shape is critical for rice production. Although significant efforts have been made to understand the nature of grain quality, a comprehensive molecular understanding of these phenotypes remains elusive. For example, "Yangzhou fried rice," a popular food cooked with long grain rice, has better palatability and morphology than short grain rice (Fig. 16.1). Those observations lead to the conclusion that the network of starch biosynthesis and rice quality regulation is complex. In this chapter we summarized what is presently known about the inheritance and molecular basis of grain quality characteristics and outlined the strategies for the development of high-quality rice in the future.

QTL mapping has been extensively and successfully applied to clone major genes that affect grain quality, especially for grain shape and ECQ. Unfortunately this approach has achieved limited success toward the cloning of minor grain quality genes. Two recently developed techniques, i.e., co-expression and association mapping, have been shown to be very useful for the identification of ECQ genes [7, 51]. It is therefore expected that fine-scale regulators that control grain quality will soon be identified by employing these methods, and subsequently cloned.

Even though rice supplies about 20 % of the world's dietary energy and is a good source of thiamine, riboflavin, and niacin [120], its protein content is much lower than other cereal crops (only 6.3–7.1 % in milling rice). More importantly, multiple micronutrient factors for human health like vitamin A, B, C, and D are defective in milled rice grain. For example, vitamin A deficiency in humans exacerbates afflictions such as diarrhea, respiratory disease, and childhood diseases such as measles [121]. Therefore, an important goal in rice grain quality research and breeding programs is to improve micronutrient content in rice endosperm. Efforts to improve vitamin A content in rice endosperm have been highly successful by the use of recombinant DNA technology to introduce 3 essential β-carotene biosynthetic pathway genes (PSY, LCY, CRTL) into the rice genome [121]. In addition, rice endosperm protein content has been significantly increased by transformation of the β-phaseolin seed storage protein gene from common bean [122, 123]. These examples demonstrate that it is possible to significantly improve rice endosperm grain quality and opens the door to engineer additional micronutrient and protein enhancements in the future.

With the world's population expected to increase from 7 to 9 billion inhabitants by 2050, rice breeders have been challenged to produce

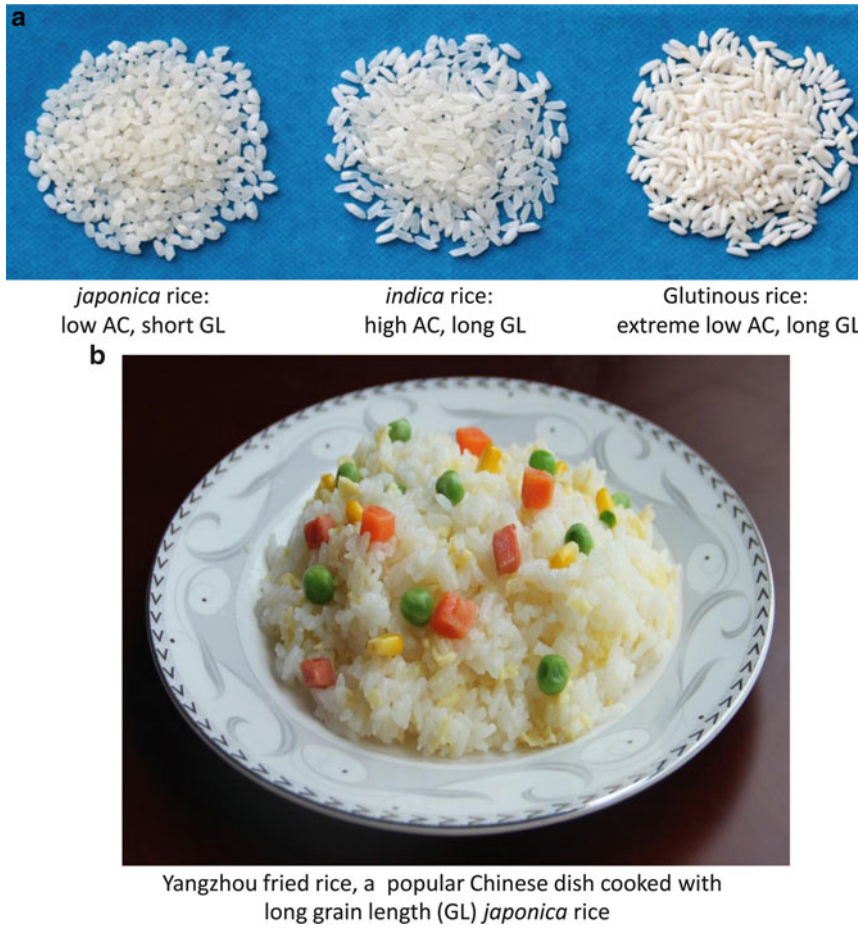

Fig. 16.1 (**a**) Three major types of rice showing their different morphologies and properties. (**b**) Yangzhou fried rice, a popular Chinese dish cooked with long grain length (GL) *japonica* rice

new cultivars that can grow with less water, fertilizer, and pesticides and have doubled yields. This challenge must also account for global warming. Preliminary data has shown that high temperatures have adverse effects on rice productivity and quality [124–128], such as the decreased brown rice rate and milling rice rate. The comparison of grain quality gene expression patterns under high and low temperatures showed that the grain quality deterioration pathway may proceed through increased sucrose synthase activity and different SSRG gene expression [127, 129]. Therefore, it is extremely important to investigate the molecular mechanisms of how high temperature signal transduction affects grain quality, which in turn will facilitate the development of new elite cultivars that have higher productivity, better quality, richer nutrients, and greater adaptation to global climate changes.

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Heterosis

17

Dan Zhu and Qifa Zhang

1 Introduction

The large-scale adoption of hybrid rice in China over the past 4 decades, and in India, Bangladesh, Vietnam, and other Asian countries in recent years, has greatly contributed to improved food availability and farmers' profitability in these countries [1]. The superior performance, or heterosis, of elite hybrids is usually featured by two important characteristics. First, elite hybrids possess greatly elevated yield potential. As much as 100 % or greater mid-parent heterosis (=F_1 – MP, where MP is the mean of the parents) and over 40 % high-parent heterosis (=F_1 – HP, where HP is the higher parent value) have been frequently observed in experimental plots [2–5]. It has been estimated that hybrids can outyield conventional cultivars by 30–40 % in production fields [6]. Second, elite hybrids often show wider adaptability, due to more resistance to biotic and abiotic stresses than inbreds, and thus perform more stably.

Advances in molecular marker technology over the past 20 years have led to significantly increased efforts aimed at understanding the biological mechanisms of heterosis in rice. In this chapter, we will summarize the major progress in heterosis research and offer our perspective for future studies.

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2 Molecular Marker Polymorphisms and Hybrid Performance in Rice

Considerable efforts were made in rice to evaluate correlations between molecular marker polymorphisms and hybrid performance and heterosis, especially in the early days of molecular marker work, with the hope to predict heterosis based on marker information. These studies made a large number of crosses utilizing a wide range of germplasms. The first study in this series was reported by Zhang et al. [2, 3] who made a set of diallel crosses of eight parental lines including three restorer lines and five maintainers of popular male sterile lines presenting a significant portion of the *indica* hybrid rice germplasms in the early 1990s (Table 17.1). Twenty-eight F_1s and eight parental lines were examined in a replicated field trial for yield, number of tillers per plant, grain weight, straw weight, and biomass, based on which mid-parent and high-parent heteroses were estimated. It was observed that high-level heterosis was common among these crosses: ≥100 % mid-parent and ≥40 % high-parent heteroses were observed in many F_1s, including some crosses between main-

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Table 17.1 Correlations of general (GH) and specific (SH) heterozygosity with hybrid performance and heterosis in four sets of diallel crosses based on data presented by Zhang et al. [2–4] and Zhao et al. [5]

Traits	Tillers per plant	Grains per panicle	Grain weight	Grain yield
Hybrid parents				
GH and performance	0.13	0.13	0.53**	0.36
GH and heterosis	0.49**	0.54**	0.30	0.56**
SH and performance	0.10	0.18	0.70**	0.48**
SH and heterosis	0.35	0.71**	0.25	0.77**
Indica sets				
GH and performance	−0.32	0.47**	0.37*	0.43**
GH and heterosis	0.17	0.24	0.14	0.34*
SH and performance	0.27	0.57**	0.61**	0.44**
SH and heterosis	0.30	0.32	0.54**	0.45**
Japonica sets				
GH and performance	0.12	0.42**	−0.08	0.48**
GH and heterosis	0.17	0.12	−0.04	0.17
SH and performance	0.52**	0.68**	0.26	0.59**
SH and heterosis	−0.03	0.13	−0.05	0.09
Intra- and inter-subspecies crosses				
Whole set of 55 crosses				
GH and performance	−0.14	0.26	0.01	0.19
GH and heterosis	0.21	0.22	0.15	0.42**
SH and performance	−0.42**	0.17	0.13	0.47**
SH and heterosis	0.08	0.22	0.11	0.09
Within *indica*				
GH and performance	−0.55	0.44	0.25	0.20
GH and heterosis	0.11	0.22	0.49	0.37
SH and performance	−0.36	0.84**	0.10	0.26
SH and heterosis	−0.16	0.33	0.32	0.33
Within *japonica*				
GH and performance	0.28	−0.15	0.18	0.43
GH and heterosis	0.10	0.02	0.03	0.41
SH and performance	0.51*	−0.56*	0.54*	0.65**
SH and heterosis	0.15	−0.23	0.24	0.52*
Between subspecies				
GH and performance	−0.05	0.19	−0.08	0.20
GH and heterosis	0.27	0.10	0.18	0.24
SH and performance	−0.21	0.29	−0.12	0.32
SH and heterosis	0.00	0.27	−0.08	−0.11

*, ** Significant at $P = 0.05$ and 0.01, respectively

tainer lines. The parental lines were genotyped using 152 molecular markers including restriction fragment length polymorphisms (RFLPs) and simple sequence repeats (SSRs), based on which the maker genotypes of the F_1s were deduced. The authors defined two types of heterozygosity based on maker polymorphisms: general heterozygosity which was calculated using all polymorphic markers employed in the survey and specific heterozygosity which was based on positive markers that detected highly significant effects on the traits. Correlations were mostly low between general heterozygosity and F_1 performance and heterosis. In contrast, very high correlations were detected between mid-parent heterosis and specific heterozygosity, especially for yield, number of grains per panicle, and biomass. The authors suggested that such high level of

Table 17.2 Correlations of F_1 heterozygosity with hybrid performance and heterosis of the three traits in the diallel set of US southern long-grained rice based on data of Saghai Maroof et al. [7]

Trait	Hybrid performance	Heterosis
Head rice percentage	–0.610**	0.198
Total head rice	0.856**	0.577**
Total rough rice	0.873**	0.462*

*, ** Significant at $P = 0.05$ and 0.01, respectively

correlations may have practical utility in predicting heterosis.

To investigate the extent to which the correlations may hold across different types of germplasm, Zhang and his associates performed similar studies using three different sets of rice genetic materials (Table 17.1). The first set consisted of nine *indica* varieties from three Asian countries with representatives of landraces, primitive cultivars, historically important cultivars, modern elite cultivars, and parents of superior hybrids [4]. The second set, made up of 11 *japonica* varieties from four countries, also represented a wide range of *japonica* rice varieties including landraces, cultivars, and hybrid parents [4]. The third set included eight lines commonly used in the US southern long-grain rice-breeding programs [7] (Table 17.2). The lines in each set were also intermated in all possible pairs following a diallel crossing scheme resulting in 36 crosses in the first set, 55 crosses in the second set, and 28 crosses in the third set. Again all the hybrids and parents were evaluated for yield traits in replicated field trials, and the parents were genotyped using molecular markers.

The amounts of correlations between marker heterozygosity and trait performance and heterosis varied drastically with traits and the sets of germplasms (Table 17.1). In the first and second sets, grain yield per plant, number of tillers per plant, number of grains per panicle, and grain weight were measured. In the first set of *indica* varieties, correlations of general heterozygosity with both performance and heterosis were low to intermediate in general. Correlations of general heterozygosity with hybrid performance appeared to be higher than those with heterosis, and correlations calculated using specific heterozygosity were slightly higher than those based on general heterozygosity. In the second set of *japonica* germplasm, little correlation was detected between heterosis and heterozygosity (either general or specific). Intermediate correlations were observed between hybrid performance and heterozygosity, and correlations based on specific heterozygosity were higher than those using general heterozygosity.

In the third set of the US long-grain rice, the traits evaluated were total rough rice, total head rice, and head rice percentage (Table 17.2). Very large positive correlations were detected between general heterozygosity and hybrid performance for total rough rice and total head rice. Intermediate negative correlation was detected between general heterozygosity and head rice percentage. Correlations of general heterozygosity with heterosis were much lower than those with hybrid performance. Specific heterozygosity was not used in the calculation because a majority of the markers appeared to be positive for total rough rice and total head rice.

There are two subspecies of the cultivated rice, *indica* and *japonica*. However, all the above mentioned studies used intra-subspecific crosses because inter-subspecific hybrids are mostly sterile. In order to investigate heterosis in inter-subspecific hybrids, Zhao et al. [5] utilized rice varieties containing wide compatibility gene(s), which could produce fertile hybrids in inter-subspecific crosses [8]. This study used 11 parental lines including five *indica* and six *japonica* varieties, in which all the *japonica* and one *indica* parents were introgressed with the wide compatibility gene(s). These lines were intermated resulting in a diallel set including 10 *indica*×*indica*, 15 *japonica*×*japonica*, and 30 *indica*×*japonica* crosses (Table 17.1). Like in the other studies, the F_1s and their parents were field tested for agronomic traits, and the parental lines were genotyped using molecular markers. Inter-subspecific hybrids showed better performance and higher heterosis than intra-subspecific hybrids. Correlations of marker heterozygosity with hybrid performance and heterosis were higher in *japonica* crosses than in *indica* crosses. Very little correlation was detected in the inter-subspecific crosses.

These results indicated that correlations between genome heterozygosity and hybrid performance and heterosis are quite complex. The situation in each data set is different from other data sets, depending on the germplasm included in the studies. Regardless of the biological basis of such complexity, there may not be a simple correlation between molecular maker heterozygosity and hybrid performance or heterosis that can be used for predicting heterosis in breeding programs across different germplasms.

3 Genetic Basis of Heterosis Analyzed Using Experimental Populations

A major series of efforts in rice heterosis study is the investigation of genetic basis of heterosis using different experimental populations. The common goal of these studies was to estimate the genetic components including dominance, overdominance, and epistasis in rice crosses for testing the classical genetic hypotheses of heterosis (Fig. 17.1).

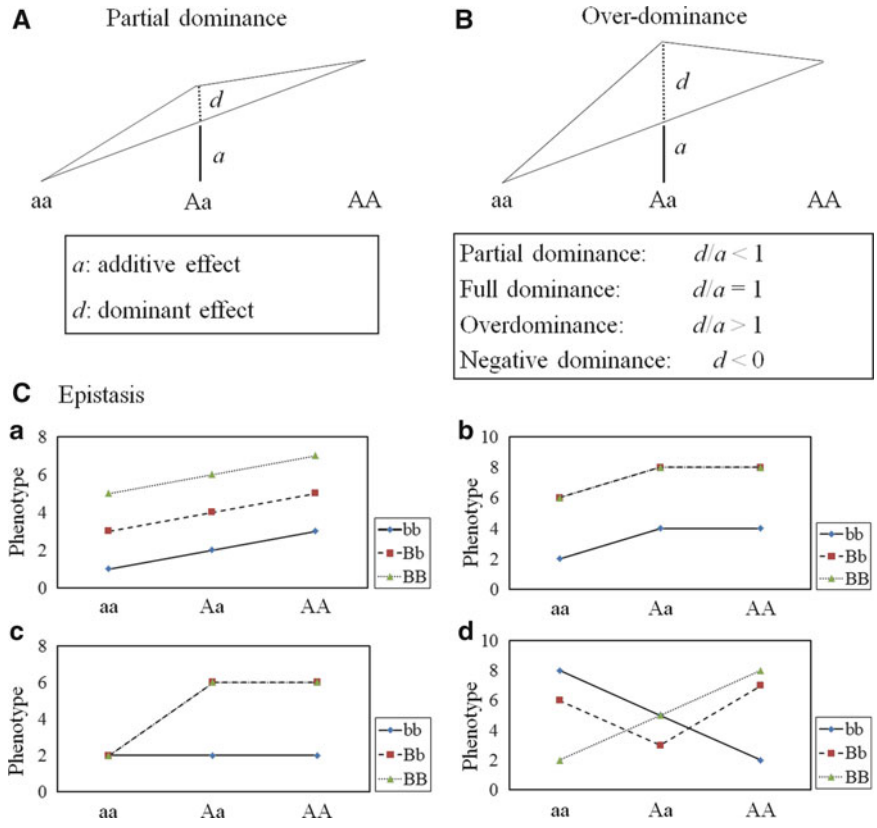

Fig. 17.1 Illustrations of models for the genetic basis of heterosis. (**A**) Dominance. (**B**) Overdominance. (**C**) Epistasis. Hypothetical patterns of phenotypes resulting from the various two-locus genotypes. Epistasis is defined as deviation from additivity between loci. (**a**) Pure additive effects within and between loci (no epistasis), (**b**) additive effects between and dominance within loci (no epistasis), (**c**) complementary dominance effects between loci (epistasis), and (**d**) complex interactions between loci (epistasis)

3.1 The 9024/LH422 Cross

Xiao et al. [9] reported a study in which a cross was made between two inbred lines—9024 (*indica*) and LH422 (*japonica*). A set of 194 recombinant inbred lines (RILs) was obtained by single seed decent to F_7, and each of the RILs was backcrossed to the two parents. The progeny materials including 388 backcrosses, 194 F_8 RILs, two parents, and the F_1 were phenotyped for 12 quantitative traits including yield and yield components. A genetic map was constructed using 141 RFLP markers. Using these data they detected a total of 37 quantitative trait loci (QTLs) in the two backcross populations. Twenty-seven QTLs were detected in only one of the backcross populations. In 82 % of these cases, the heterozygotes were superior to the respective homozygotes. The ten remaining QTLs were detected in both backcross populations, and the heterozygotes had phenotypes falling between those of the two homozygotes. There was very little correlation between performance of most traits and overall genome heterozygosity. The authors interpreted the results as suggesting that dominance complementation is the major genetic basis of heterosis in rice.

3.2 The Teqing/Lemont Cross

Li et al. [10] and Luo et al. [11] made a cross between Teqing, a high-yielding *indica* cultivar from China, and Lemont, a *japonica* cultivar from the USA with wide compatibility, from which 254 RILs were obtained by single seed descent. The RILs were crossed to the two parental lines, resulting in two backcross hybrid populations, 177 hybrids from the crosses of RILs with Teqing ($TQBCF_1$), and 172 hybrids from the crosses of the RILs with Lemont ($LTBCF_1$). In addition, 192 and 187 hybrids were obtained by testcrossing the RILs with Zhong 413 (a Chinese restorer line with wide compatibility) and IR64 (a cultivar widely used in Southern and Southeastern Asian countries), respectively. The RILs, the two BC_1F_1 populations ($LTBCF_1$ and $TQBCF_1$), the two testcross populations ($Z413TCF_1$ and $IR64TCF_1$), the parents (Lemont and Teqing), and their F_1 were evaluated in field experiments at two locations for biomass, grain yield, and other traits that are components of yield, including panicles per plant, grains per panicle, and grain weight. Data for 179 RFLP markers and four morphological markers were obtained to construct the linkage map, which was used for mapping heterosis.

Heterosis for yield and all three yield component traits was observed in the backcross and testcross populations. On the basis of mid-parent heterosis, the $IR64TCF_1$ population showed the highest heterosis in yield, followed by the $LTBCF_1$ population (second), the $TQBCF_1$ (third), and the $Z413TCF_1$ the lowest. However, the relative levels of heterosis of the component traits differed from one cross to another. Within each of the F_1 populations, individual hybrids varied considerably in their performance and heterosis. Most backcross or testcross hybrids showed highly significant positive heterosis, and hybrids showing significant negative heterosis for these traits were also observed although much less frequent in all four F_1 populations.

The trait performance and mid-parent heterosis of individual backcross and testcross F_1 hybrids were used to identify QTLs contributing to heterosis. Large numbers of main-effect QTLs and epistatic QTLs were resolved for performance and heterosis of the four traits in the various populations. Additive and dominance effects were also estimated for each of the QTLs. Using the trait number of grains per panicle for illustration, four main-effect QTLs affecting this trait (one in location and three in the other location) were identified in the RILs. Eleven QTLs affecting F_1 mean values and/or heterosis were detected in the BC or testcross F_1 populations. Of these QTLs, two showed additive effects as they were detectable only by the F_1 mean values, one detected in the $LTBCF_1$s appeared to be dominant, and the remaining eight appeared to be overdominant since the QTL effects estimated from heterosis values were equal to or greater than their effects estimated from F_1 mean values.

In addition, 19 pairs of digenic interactions were identified affecting this trait from the F_1

mean and/or heterosis of the backcross and testcross populations, including six in the $LTBCF_1$s, five in the $TQBCF_1$s, five in the $Z413TCF_1$s, and three in the $IR64TCF_1$s, respectively. A majority of the epistatic QTLs represent dominance-by-dominance interactions.

The authors made two conclusions from these analyses. First, most QTLs associated with heterosis appeared to be involved in epistasis. Second, most QTLs contributing to heterosis appeared to be overdominant. These observations implicate epistasis and overdominance as the major genetic basis of heterosis, within the limit of this set of experimental materials.

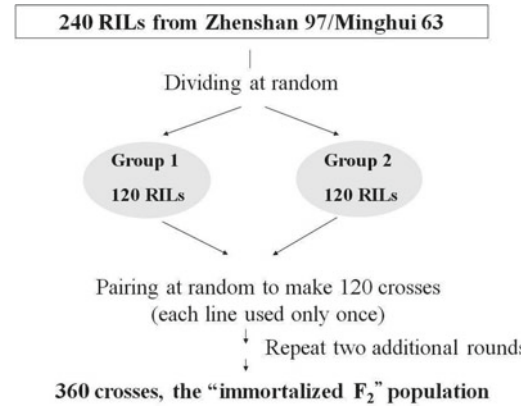

Fig. 17.2 Construction of an immortalized F_2 population

3.3 The Zhenshan 97/Minghui 63 Cross

Shanyou 63, a cross between two *indica* lines Zhenshan 97 and Minghui 63, is an elite hybrid that has been widely planted in rice production over the past 3 decades. The area planted with Shanyou 63 reached at 6.7 million ha in its peak period in late 1980s and early 1990s which accounted for over 25 % of rice area in China during that time. The major characteristics of this hybrid are high and stable yield.

To illustrate the genetic basis of heterosis in this hybrid, Yu et al. [12] made a cross between Zhenshan 97 and Minghui 63 and used 250 F_3 families as the experimental population; each was derived from bagged seeds of a single F_2 plant. A molecular linkage map was constructed with 150 segregating loci covering the entire rice genome. Data for yield and three yield component traits were collected over 2 years from replicated field trials of the $F_{2:3}$ families, the parents, and the F_1. It was estimated that genotypic variations explained from about 50 % to more than 80 % of the total variation. A total of 32 QTLs were detected for the four traits; 12 were observed in both years and the remaining 20 were detected in only 1 year. Overdominance was observed for most of the yield QTLs and a few yield component trait QTLs. Correlations between marker heterozygosity and trait values were low, indicating that the overall heterozygosity made little contribution to heterosis. Digenic interactions, in the forms of additive-by-additive, additive-by-dominance, and dominance-by-dominance, were frequent and widespread in this population. The interactions involved large numbers of marker loci, most of which were not detected on a single-locus basis; many interactions among loci were detected in both years. Based on these results, the authors concluded that epistasis plays an important role as the genetic basis of heterosis.

To further this study, Hua et al. [13, 14] employed a new experimental design that produced an "immortalized F_2" population by using 240 F_9 RILs derived by single seed descent from the cross between Zhenshan 97 and Minghui 63. In this design (Fig. 17.2), crosses were made between the RILs chosen by random permutations of the 240 RILs. In each permutation round, the 240 RILs were randomly divided into two groups, and the lines in the two groups were paired up at random without replacement to provide parents for 120 crosses. This procedure was repeated three times, resulting in a population of 360 crosses. This population resembles an F_2 population in that the compositions and frequencies of single- and multi-locus genotypes are the same as those in an F_2 population. Field trials of the immortalized F_2 population were conducted, in which the hybrids and the corresponding parental RILs were planted in the same plots. Traits examined included yield per plant, tillers per plant, grains per panicle, and grain weight.

Molecular marker linkage maps consisting of 231 polymorphic loci were constructed for the RIL population, as well as the immortalized F_2 population with the genotype data of each cross deduced from the parental RILs.

Two analyses were performed using this data set. In the first analysis using the performance data, Hua et al. [13] resolved the genetic effects into individual components and assessed relative performance of all the genotypes at both single- and two-locus levels. Single-locus analysis detected 40 QTLs for the four traits. Dominance effects for about half of the QTLs were negative, resulting in little "net" positive dominance effect. Correlation between genotype heterozygosity and trait performance was low. Large numbers of digenic interactions, including additive-by-additive, additive-by-dominance, and dominance-by-dominance, were detected for all the traits, with additive-by-additive as the most prevalent interactions. A very interesting observation was that complementary two-locus homozygotes, rather than heterozygotes, performed the best among the nine genotypes of many two-locus combinations, despite the fact that the population was derived from a highly heterotic cross. While cumulative small advantages over two-locus combinations may partly explain the genetic basis of heterosis of the hybrid as double heterozygotes frequently demonstrated marginal advantages, double heterozygotes were never the best genotypes in any of the two-locus combinations. The results suggested that heterozygotes were not necessarily advantageous for trait performance even among genotypes derived from such a highly heterotic hybrid.

In the second analysis, Hua et al. [14] calculated the mid-parent heterosis value for each cross for all the traits and used the data to analyze the genetic basis of heterosis. They modified slightly the composite interval mapping method [15, 16] to detect heterotic loci by assuming that the two homozygotes of each locus did not contribute to heterosis, and a locus was considered to be heterotic loci only when showing significant difference in heterosis between the heterozygote and the mean of the two homozygotes. Heterotic effects were detected at 33 loci for the four traits. The heterotic loci showed little overlap with QTLs for trait performance detected by Hua et al. [13], suggesting that heterosis and trait performance may be conditioned by different sets of loci. Large numbers of digenic interactions were resolved using two-way ANOVA and confirmed by permutation tests. All kinds of genetic effects, including partial, full, and overdominance at single-locus level, and all three forms of digenic interactions (additive-by-additive, additive-by-dominance, and dominance-by-dominance) contributed to heterosis in the immortalized F_2 population, indicating that these genetic components were not mutually exclusive in the genetic basis of heterosis. Heterotic effects at the single-locus level, in combination with the marginal advantages of double heterozygotes, due to dominance-by-dominance at the two-locus level, could explain the genetic basis of heterosis in Shanyou 63. The authors suggested that these results may help reconcile the century-long debate concerning the genetic basis of heterosis.

These analyses demonstrated a number of distinct advantages of the use of an immortalized F_2 population for the genetic analyses of both trait performance and heterosis. (1) All the possible genotypes are observed and their proportions are similar to those in an F_2 population (i.e., 1:2:1 for single-locus genotypes and 1:2:1:2:4:2:1:2:1 for two-locus combinations). Thus, such a population is genetically as informative as an F_2 population, which allows for the detection of the genetic components such as additive and dominance effects at the single-locus level and interactions of multiple loci. (2) Instead of only one individual per genotype represented in an ordinary F_2 population, each genotype in this population is represented by as many plants as the researcher desires, thus permitting replicated trials. The whole population can be recreated when needed, either in exactly the same way or by different permutation schemes, thus allowing for trials in multiple years and locations. (3) The molecular marker data need to be collected from only the 240 RILs no matter how many crosses are included in the population. (4) Plants used for measuring heterosis are hybrids rather than progenies of self-fertilization.

This may provide particular value for heterosis analyses, if epigenetic changes associated with hybridization are involved in heterosis or inbreeding depression. (5) Most importantly, it provides opportunities for mapping and genetic analysis of heterosis per se, rather than analyses based on performance measurements of the trait. (6) The immortalized F_2 population can be created using any segregating homozygous populations such as RILs and doubled haploid lines, thus may have general applications for complete resolutions of genetic components of quantitative traits and heterosis. It should be noted that the same mating scheme was reported in mice genetic studies which was referred to as a recombinant inbred intercross [17].

Recently, Xie et al. [18] genotyped the RILs by population sequencing with a parent-independent method they developed for constructing ultra-high density linkage maps composed of high-quality SNPs. The method involved the following steps. First, all potential SNPs were identified to obtain drafts of the parental genotypes using a maximum parsimonious inference of recombination, making maximum use of SNP information found in the entire population. Second, high-quality SNPs were identified by filtering out low-quality ones with permutations involving resampling of windows of SNPs followed by Bayesian inference. Third, RILs in the mapping population were genotyped using the high-quality SNPs assisted by a hidden Markov Model.

Employing this method, an ultrahigh-density linkage map composed of bins of high-quality SNPs was constructed using 238 of the RILs mentioned above with 0.055× genome sequence per line, followed by QTL analysis of yield and three yield component traits [19]. Compared to QTL mapping based on RFLPs/SSRs, the SNP bin map detected more QTLs with precise map locations, thereby demonstrating advantages in the detecting power and resolution relative to the RFLP/SSR map.

Using this map, Zhou et al. [20] reanalyzed the data of yield and yield component traits of the immortalized F_2 population by calculating single-locus and epistatic genetic effects in the whole genome and identified components pertaining to heterosis of the hybrid. The results showed that the relative contributions of the genetic components varied with traits. Overdominance/pseudo-overdominance is the most important contributor to heterosis of yield, number of grains per panicle, and grain weight. Dominance-by-dominance interaction is important for heterosis of tillers per plant and grain weight and also has roles in yield and grain number. Single-locus dominance has relatively small contributions in all the traits. The results again suggested that cumulative effects of these components may adequately explain the genetic basis of heterosis in the hybrid.

4 Attempts to Characterize Molecular Basis of Heterosis

4.1 Gene Expression and Heterosis

Xiong et al. [21] analyzed relationships of differential gene expression in leaves with heterozygosity and heterosis of six agronomic traits in a rice diallel cross involving eight elite rice lines previously described by Zhang et al. [2, 3] by assaying patterns of differential gene expression in hybrids relative to their parents using differential display. Although the analysis revealed several interesting patterns of differential expression, there was very little correlation between patterns of differential expression and the F_1 means for all six agronomic traits. Differentially displayed fragments that occurred only in one parent but not in the other parent or F_1 in each of the respective crosses were positively correlated with heterosis and heterozygosity. While fragments that were detected in F_1s, but in neither of the respective parents, were negatively correlated with heterosis and heterozygosity. Other patterns of differential fragments were not correlated with heterosis or heterozygosity. It was suggested that the relationships between the patterns of differential expression and heterosis observed in this study were not consistent with expectations based on dominance or overdominance hypotheses.

The advent of DNA microarray technology and complete sequencing of the rice genome made it possible to compare whole-genome expression profiles of the parents and hybrid for many of the populations described above. For example, Huang et al. [22, 23] analyzed gene expression profiles of the hybrid Shanyou 63 and its respective parents Zhenshan 97 and Minghui 63 using young seedlings and panicles at three stages of young panicle development with a cDNA microarray consisting of 9,198 expressed sequence tags (ESTs). Wei et al. [24] performed a transcriptomic analysis of the hybrid LYP9, the most widely cultivated two-line hybrid in China, and its two parents 93-11 and PA64s. The analysis employed a whole-genome oligonucleotide microarray based on known and predicted *indica* rice genes using seven tissues from developing leaves and panicles. Song et al. [25] compared expression profiles of a hybrid Liangyou 2186 and its parents using serial analysis of gene expression (SAGE). A common finding from this type of analyses is that large numbers of genes distributed throughout the genome were found to be differentially expressed in the hybrids compared with the parents, and the genes could be classified into various categories according to their biological functions. However, the primary difficulty of interpreting such data sets is how to associate the differentially expressed genes with trait performance, especially with heterosis of agronomically important traits.

4.2 Epigenetic Modification and Heterosis

DNA methylation has been known to play an important role in the regulation of gene expression in eukaryotes. Xiong et al. [26] developed a technique referred to as "methylation-sensitive amplified polymorphism" to assess the extent and pattern of cytosine methylation in the rice genome. They compared patterns of cytosine methylation in Shanyou 63 and the two parents Zhenshan 97 and Minghui 63 using seedlings and flag leaves. Differential patterns of cytosine methylation in the hybrid relative to the parents were detected including increased methylation in the hybrid compared to the parents at some recognition sites, while decreased methylation in the hybrid at other sites. These results suggested possible relation of DNA methylation and heterosis.

Recently He et al. [27] performed a genome-wide analysis of epigenetic and transcriptional trends using 9311 (*indica*), Nipponbare (*japonica*), and their reciprocal hybrids by assaying their epigenome, mRNA, and small RNA transcriptomes. They identified differential epigenetic modifications that are correlated with changes in transcript levels among hybrids and parental lines and distinct patterns in gene expression and epigenetic modifications in reciprocal hybrids. They also observed a high correlation of allelic bias of epigenetic modifications or gene expression in reciprocal hybrids with their differences in the parental lines. The abundance of distinct small RNA size classes differed between the parents, and more small RNAs were downregulated than upregulated in the reciprocal hybrids.

Again the difficulty with interpreting this data is how to establish a relationship between these epigenetic modifications and the phenotype, especially with respect to heterosis of agronomically important traits.

4.3 Hormonal Basis of Heterosis

All the biological processes underlying growth and development of plants are regulated by complex interactions of phytohormones. Although it is easy to speculate the involvement of hormones in the superior performance of hybrids, experimental evidence demonstrating a role in phytohormone regulation of heterosis is surprisingly meager.

In maize (*Zea mays*), Rood et al. [28] analyzed the responsiveness of F_1 hybrids and their inbred parents of diallel combinations to the exogenous application of GA3 and endogenous levels of GAs. They found that inbreds were more responsive than the hybrids to the exogenous GA3, while the hybrids had higher concentrations of endogenous GAs than their parental inbreds. They concluded that the increased endogenous concentration of GA in the hybrids

could provide a phytohormone basis for heterosis for shoot growth.

In rice Ma et al. [29] investigated the possible relationship between GA signaling and heterosis for seedling growth and development. They performed a quantitative analysis of endogenous gibberellin (GA) contents and expression profiling of the GA metabolism and signaling genes using an incomplete diallel set of 3×3 crosses and the six parents. They detected significant positive correlations between the contents of some GA species and performance and heterosis based on shoot dry mass in seedling shoots at 20 days after sowing. Expression analyses of GA-related genes by real-time PCR revealed that 13 out of the 16 GA-related genes examined exhibited significant differential expression among the F_1 hybrid and its parents. Expression levels of nine genes in the hybrids displayed significant positive correlations with the heterosis of shoot dry mass. These findings provided supporting evidence for GAs playing an important regulatory role in heterosis for rice seedling development.

5 The Genic Basis of Heterosis

Ultimate understanding of the biological basis of heterosis depends on molecular characterization of genes involved in heterosis. In tomato, the flowering gene *SINGLE FLOWER TRUSS* (*SFT*) was reported to produce heterosis for yield via overdominance in fruit number [30]. A large number of genes for yield and yield component traits have been cloned in rice [31], and genetic effects of genomic regions on agronomic performance have also been analyzed using NILs in a number of cases. We have listed examples from the literature which may provide some feeling about the genetic effects of the genes on heterosis (Table 17.3).

Two of the cloned genes, *Ghd7* and *GS5*, are most relevant to the heterosis of the Shanyou 63 hybrid (Table 17.3). *Ghd7*, cloned by Xue et al. [32], controls two interrelated panicle traits, both of which showed heterosis. NIL(mh7), an NIL homozygous for the Minghui 63 allele in the background of Zhenshan 97, produced 132.9 grains per panicle, and NIL(zs7), homozygous for the Zhenshan 97 allele which completely lacked *Ghd7*, produced 65.3 grains per panicle, while the heterozygote NIL(het) produced 114.9 grains per panicle, amounting to 15.9 % mid-parent heterosis. A similar level of heterosis (14.5 %) was observed when evaluated using number of spikelets per panicle. Thus, this locus clearly showed partial dominance.

The other example is *GS5*, a gene for grain width [33], which was cloned using genetic materials derived from two varieties, Zhenshan 97 and H94; the latter has identical sequence to Minghui 63 in the *GS5* region. An analysis using NILs having the Zhenshan 97 genetic background showed that heterozygote had 3.0 % advantage in grain width relative to the mean of the two homozygotes (Table 17.3), which again indicates partial dominance.

Data from phenotyping a number of NILs (Table 17.3) showed that although the NILs were extracted from crosses of very diverse germplasm, the heterozygotes always showed partial to nearly full dominance regardless of the traits, suggesting the prevalence of partial dominance that may contribute to heterosis.

However, the available information is still limited because the number of genes cloned so far for agronomic performance is small, and their effects in hybrids have not been measured. Genetic materials such as reciprocally introgressed NILs may be developed and employed for characterizing regions showing significant effects on heterosis at both individual loci as well as epistatic interactions.

6 Future Perspectives

Although a wide range of studies have been conducted to investigate heterosis, a comprehensive understanding of the biological mechanisms of this extremely important trait lies in systematic characterization of its genetic basis. In particular, to be relevant to heterosis in crop hybrids, studies should focus on the genetic dissection of heterosis in agronomic performance such as yield and yield component traits using advance mapping populations constructed with elite hybrids.

Table 17.3 The cloned genes and QTLs and their genetic effects relevant to heterosis

Gene†/QTL‡	Genotype	Grain length	Grain width	Grains per panicle	Spikelets per panicle	Reference
Ghd7†	NIL(zs7)			65.3 A	130.6 A, a	[32]
	NIL(het)			114.9 B	198.8 B, b	
	NIL(mh7)			132.9 C	216.6 B, c	
GS5†	GS5(H94)		2.92 A			[33]
	GS5(het)		3.13 B			
	GS5(ZS97)		3.16 B			
Gn1a†	NIL(*gn1a*)			164		[42]
	NIL(het)			180		
	NIL(*Gn1a*)			198		
GS3†	NIL(c7)	7.57 A				[43]
	NIL(het)	8.23 B				
	NIL(zs97)	9.02 C				
OsSPL16†	NIL(GW8)	8.14	2.64			[44]
	NIL(het)	8.53	2.46			
	NIL(gw8)	8.81	2.24			
SPP3b‡	NIL(MH63)				117.7	[45]
	NIL(het)				123.5	
	NIL(TQ)				141.5	
qSPP1‡	NIL(ZS97)			82.5 A	98.5 A	[46]
	NIL(het)			109.9 B	136.7 B	
	NIL(HR5)			117.7 B	144.1B	
qSPP2‡	NIL(ZS97)			86.6 A	100.7 A	[46]
	NIL(het)			103.8 B	126.8 B	
	NIL(HR5)			106.6 B	131.6 B	
qSPP3‡	NIL(ZS97)			86.6 A	114.1A	[46]
	NIL(het)			119.9 B	153.0 B	
	NIL(HR5)			132.1 C	166.6 C	
qSPP7‡	NIL(ZS97)			76.5 A	108.9 A	[46]
	NIL(het)			108.3 B	160.9 B	
	NIL(HR5)			112.4 B	172.1 B	
qSPP8‡	NIL(ZS97)			78.9	94.0	[47]
	NIL(het)			117.8	136.9	
	NIL(HR5)			129.0	150.6	
SPP1‡	NIL(ZS97)				160.6	[48]
	NIL(het)				183.2	
	NIL(TQ)				204.7	
gpa7‡	NIL(SIL040)			99.4 a		[49]
	NIL(het)			157.3 b		
	NIL(Guichao 2)			161.1 b		
qGL7‡	NIL(Chuan 7)	6.8			152 a	[50]
	NIL(het)	7.3			115 b	
	NIL(Nanyangzhan)	7.4			117 b	
PBN6‡	NIL(Sasanishiki)				89.7 a	[51]
	NIL(het)				95.4 b	
	NIL(Habataki)				98.1 c	
qSN9.1‡	NIL(Hwaseongbyeo)			109.6 a	115.8 a	[52]
	NIL(het)			114.2 b	120.7 b	
	NIL(*O. rufipogon*)			130.4 c	138.7 c	

A, B, and C: ranked by Duncan test at $P = 0.01$. a, b, and c: ranked by Duncan test at $P = 0.05$

†The genes related to yield and yield component traits

‡The QTLs mapped through NILs for yield and yield component traits

Innovative experimental designs allowing for complete dissection of the genetic components pertinent to heterosis should be employed in the analysis, and genotyping methods utilizing new sequencing technologies allowing for unambiguous analysis and full recovery of genetic effects in any genomic region should also be applied. As the first step, it is imperative to obtain a genetic framework at the whole-genome level which should provide mapping information and quantitative assessment for all heterotic genetic effects, including dominance and overdominance at individual loci and epistasis involving dominance-by-dominance interactions, and their relative contributions to heterosis of the traits. NILs developed using elite hybrids are critical for precise quantitative analysis of heterotic genetic effects in a homogenous genetic background for individual genes of interest. For eventual understanding, efforts should be made to molecularly identify and clone the genes and illustrate the molecular mechanisms of how their effects are related to heterosis of the traits. Understanding based on dissecting individual genes should be reassembled according to the genetic framework thus providing the totality of biological characterization of heterosis at the whole system level for a particular hybrid.

Rice is rich in germplasm resources. There are 24 species in the genus *Oryza* including two cultivated species, Asian cultivated rice *O. sativa* L. and African cultivated rice *O. glaberrima* L., and 22 wild relatives [34]. Asian cultivated rice consists of two subspecies, *indica* and *japonica*, and the genomes are highly diverse at the species level featuring extensive genetic differentiation between *indica* and *japonica* subspecies [35–37]. However, widely cultivated commercial hybrids at present are exclusively *indica* and have narrow germplasm basis.

High heterosis has frequently been reported in *indica-japonica* crosses. Indeed strategies have been proposed for developing inter-subspecific hybrids [6]. However, the progress has been hindered by high sterility in the *indica-japonica* hybrids [38]. Recent genetic studies have identified a large number of loci for hybrid sterility, and several of these genes have been cloned [39, 40], which have brought hope for overcoming the hybrid sterility. Now the time has come to develop strategies for efficient utilization of germplasms across subspecies with the goal to develop inter-subspecific hybrids.

Recently Zhang [41] proposed Green Super Rice as new goal for rice improvement for further increasing productivity and sustainable production. This not only requires yield increase and quality improvement but also needs resistance to multiple insects and diseases, high nutrient use efficiency, and drought resistance so that rice production should require less input and be more environment friendly. Hybrid rice breeding as well as heterosis studies should also address those traits as their targets.

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Flowering

18

Hiroyuki Tsuji and Ko Shimamoto

1 Importance of Flowering Time Control

Flowering time, or heading date in cereal crops, is a crucial agronomic trait for the adaptation of crops to specific cultivation areas and growing seasons. The major advantages to controlling flowering are to enlarge areas of cultivation, adjust harvest time with the local climate, and establish double and triple cropping, all of which could provide higher yields in cropping locations. In rice, extensive selection of early flowering and photoperiod-insensitive cultivars enabled harvests prior to the arrival of low temperatures in the autumn, resulting in broader areas of cultivation in north-eastern Asia including Japan, Korea, and the northern area of China [16]. The flowering times of local cultivars have been more precisely adjusted to avoid preharvest sprouting under preferable humidity conditions. Early flowering cultivars in warm temperature areas are also useful for the introduction of double, or in some cases, triple cropping techniques especially in the humid and subtropical areas of Asia. In addition to classical, grain yield-oriented applications, recent demands for crops to use for biofuels have stimulated greater interest in flowering time control of some cereal crops. Rice provides an excellent model system for investigating the genetic control of flowering time through decades of achievement in flowering research [48]. The onset of flowering terminates growth of vegetative organs that are useful for biofuel production, thus controlled late flowering will result in crops that are more ideally suited for fuels. The rice genome shows extensive synteny to other cereal crops, and information about the genetic elements that control flowering time can accelerate the improvement of flowering time control in biofuel cereal crops [3]. The molecular and genetic determinants for flowering time control are, thus, particularly important targets for modern and future breeding objectives.

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2 Molecular Nature of Florigen

Photoperiodic flowering has long been considered as a systemic response throughout the plant, including day-length perception in leaves, generation of a mobile flowering signal, and its long-distance transport to the shoot apex [44]. The molecular nature of the mobile flowering signal has long been a mystery in plant science, but recent efforts to understand this systemic process revealed that Heading date 3a (Hd3a)/FLOWERING LOCUS T (FT) proteins serve as the long-sought-after florigen (Fig. 18.1a) [5, 40].

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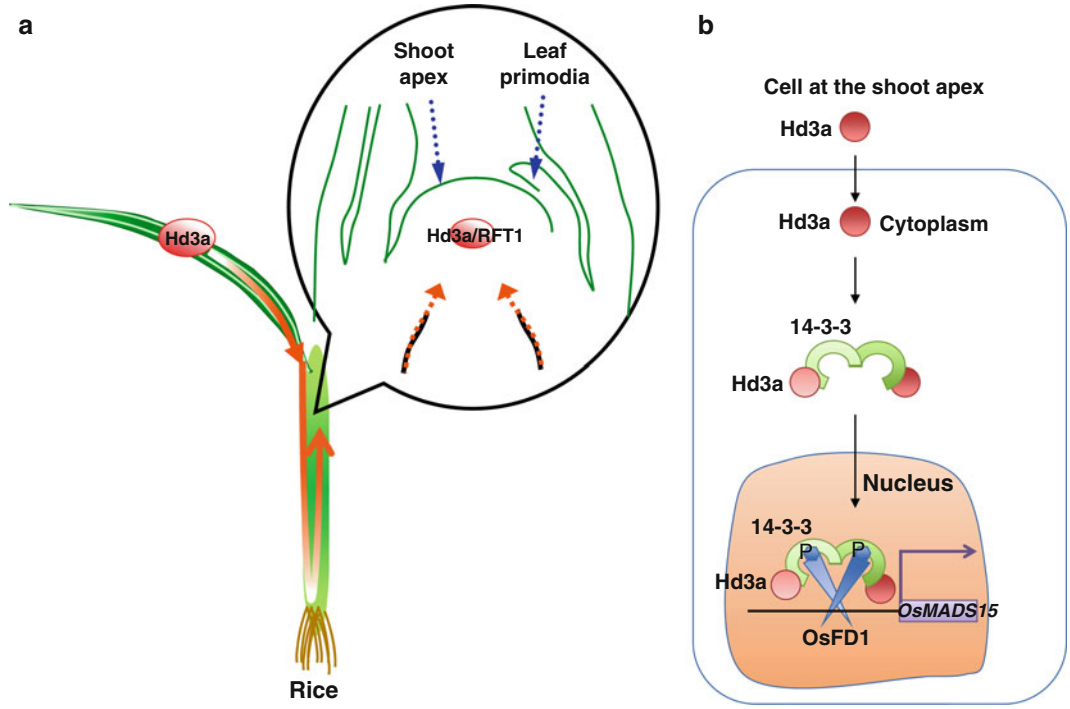

Fig. 18.1 Model of florigen function in rice. (**a**) Hd3a protein is produced in the phloem companion cells in leaf blades and is transported to the shoot apex through the phloem. (**b**) In shoot apical cells, Hd3a interacts with 14-3-3 proteins and translocates into the nucleus where Hd3a-14-3-3 interacts with OsFD1 and activates *OsMADS15* transcription, leading to flowering

The *Hd3a* gene was first identified in rice from a quantitative trait locus (QTL) affecting flowering time that promotes flowering under short-day (SD) conditions. Map-based cloning of this QTL revealed that *Hd3a* was orthologous to the Arabidopsis *FT* gene, also known as a strong activator of flowering, and encodes a protein similar to a phosphatidylethanolamine-binding protein (PEBP) with a small globular structure [23]. The precise tissue locations for *Hd3a* transcription, mRNA accumulation, and accumulation of translated protein were determined using transgenic plants expressing various reporters under the control of the *Hd3a* promoter [40]. *Hd3a* transcription was specifically active in leaf phloem tissue, and no activity was detected in the shoot apical meristem (SAM). *Hd3a* mRNA accumulated in the leaf blades, but was present at very low levels in other organs and was four orders of magnitude lower in the shoot apex than in leaf blades. Hd3a protein localization was examined using transgenic plants expressing an Hd3a-GFP fusion protein under the control of the *Hd3a* promoter. Hd3a-GFP protein was clearly detected in tissues in and around the SAM, from the stem vascular tissue to the inner corn-like region of the SAM, suggesting that Hd3a-GFP protein moves from the end of the vascular tissue to the SAM. When the Hd3a-GFP protein is expressed from two other phloem-specific promoters, Hd3a-GFP accumulated again in the SAM, confirming the mobile nature of the Hd3a protein [40]. The plants with Hd3a-GFP expression flowered earlier than wild-type (WT) plants [40], and RNAi suppression of *Hd3a* strongly delayed flowering under inductive SD conditions [24]. Altogether, Hd3a protein fulfills the requirement of a florigen, the mobile floral activator generated in the leaf under floral promoting conditions and moved up to the SAM to initiate the floral transition.

A detailed analysis of the close paralog of Hd3a, *RICE FLOWERING LOCUS T1* (*RFT1*), showed that the protein encoded by *RFT1* also acts as a florigen. *Hd3a* RNAi suppression strongly delayed flowering only in SD conditions; *Hd3a* RNAi plants flowered quite normally in LD conditions. In contrast, *RFT1* RNAi suppression specifically delayed flowering in LD conditions, but not in SD conditions. These results clearly indicate that rice florigen varies depending on the photoperiod; Hd3a functions as the SD florigen and RFT1 functions as the LD florigen [25]. The two florigen genes are regulated differentially by photoperiod, and the gene networks that govern specific induction of the two florigens diverge to confer flowering in any photoperiodic condition. When Hd3a and RFT1 activities are simultaneously eliminated by double RNAi, the rice plants never flower, indicating that flowering control of rice completely depends on the two florigens [24].

3 Florigen Activation Complex

A model from an Arabidopsis study suggested that FT protein is transported from leaves to interact with the bZIP transcription factor, FD, in the shoot apex to activate expression of a floral meristem identity gene *APETALA1* (*AP1*) [1, 51], but the precise molecular mechanism for florigen action was poorly understood when Hd3a protein was identified as a florigen. Hd3a protein interacts with a rice FD homolog, OsFD1, in a yeast two-hybrid system, but there are no direct interactions in vitro using purified proteins. Another class of Hd3a interacting proteins, 14-3-3 proteins, can directly interact with not only Hd3a but also OsFD1 in vitro, and addition of a 14-3-3 protein conferred the ability of Hd3a to form a complex with OsFD1. These findings suggest that 14-3-3 mediates Hd3a-OsFD1 interaction, and the resulting Hd3a-14-3-3-OsFD1 complex, named the florigen activation complex (FAC), is the genuine protein complex involving Hd3a and FD1 (Fig. 18.1b) [42].

Compellingly, the crystal structure of the FAC has been determined in which the nine C-terminal amino acids of OsFD1 including a phosphorylated serine residue were used. The structure suggested that FAC is hetero-hexamer composed of two molecules each of Hd3a, 14-3-3, and OsFD1. Two Hd3a monomers bind on the C-terminus of the 14-3-3 dimer, to form a W-shaped structure. The S192-phosphorylated OsFD1 C-terminus is inserted into the inner corner of the W-shaped structure, and Hd3a and OsFD1 were not in direct contact as suggested from the in vitro study. Hydrophobic side chains of Hd3a were deeply inserted to interact with the hydrophobic cavity of the 14-3-3 protein C-terminus. Hd3a R64 also interacts with the acidic lobe of the 14-3-3 protein around the above-mentioned hydrophobic cavity. The 14-3-3 protein's phospho-serine binding grooves interact with the phosphorylated serine 192 of OsFD1; this interaction resembles the canonical mode-I interaction of 14-3-3 protein and phosphorylated substrates [42].

The importance of FAC formation was examined by protoplast transient assay and transgenic plants expressing mutant versions of Hd3a or OsFD1. In rice protoplasts, transient expression of Hd3a and OsFD1 resulted in the formation of FAC by endogenous 14-3-3 proteins bridging both Hd3a and OsFD1 interactions and activated rice *AP1* expression. When Hd3a or OsFD1 is mutated to abolish 14-3-3 protein interaction, the ability to activate *AP1* expression is lost. In transgenic plants, overexpression of wild-type Hd3a can promote flowering, but overexpression of a noninteracting Hd3a mutant cannot, suggesting that the reduced affinity of Hd3a for 14-3-3 protein abolishes the accelerated flowering. Altogether, FAC formation is necessary for Hd3a to activate *AP1* expression and promote flowering [42].

4 Florigen Receptor

The next question to address is how FAC is built in the cells of the shoot apex. Detailed observation throughout the process of FAC formation indicated that this process involves dynamic changes in the subcellular localization of FAC components. Hd3a is normally present in both

cytoplasm and nucleus, 14-3-3 protein is present in the cytoplasm, and OsFD1 is localized in the nucleus, indicating different subcellular localization patterns of the three proteins. When Hd3a-14-3-3 interaction was visualized by a fluorescent complementation approach, the Hd3a-14-3-3 complex was detected mainly in the cytoplasm where 14-3-3 proteins are mainly present. Although there is no overlap in the localizations of Hd3a-14-3-3 and OsFD1 at this stage, when OsFD1 is co-expressed then the Hd3a-14-3-3 complex translocates from the cytoplasm to the nucleus to form FAC, thereby activating *AP1* transcription (Fig. 18.1b) [42].

From these observations, the receptor of florigen Hd3a can be the 14-3-3 proteins. Florigen is a plant hormone that is synthesized in the leaves and transported to the shoot apex where it induces flowering, and thus florigen requires receptors inside the cell to initiate cellular signaling to activate floral meristem identity genes. The 14-3-3 proteins are the only interacting proteins that bind with florigen directly, and florigen function is completely dependent on the ability to interact with 14-3-3 protein. Thus, Hd3a initially interacts with 14-3-3 proteins in the cytoplasm, then translocates into the nucleus to further interact with the transcription factor OsFD1 to form the FAC that is essential for promoting flowering. Based on these results, the 14-3-3 proteins are considered to act as intracellular florigen receptors [42].

5 Photoperiodic Control of Florigen Expression

From an understanding of the molecular nature of florigen and its essential role on flowering, the regulation of photoperiodic flowering is inferred ultimately as the regulation of expression of two essential florigen genes by different day lengths [45]. The crucial aspects of this regulation are SD promotion and LD suppression of florigen expression. Recent molecular genetic studies identified networks in which evolutionarily conserved components constituting the basic form of photoperiodic florigen activation were present in all of the flowering plants studied, and specifically evolved components that confer flowering in a wide range of environmental conditions in rice (and/or other cereals) (Fig. 18.2) [16, 45].

6 Evolutionarily Conserved Pathways

The evolutionarily conserved flowering module involves the GIGANTEA(GI)-CONSTANS(CO)-FT signaling cascade that was first established in the model plant Arabidopsis [22, 46]. GI, a large protein with unknown biochemical function, plays a crucial role in the circadian clock by stabilizing the central clock oscillator [21]. In the context of photoperiodic flowering, GI up-regulates expression of *CO*, encoding a B-box zinc finger transcription factor with a C-terminal conserved CCT (CONSTANS, CONSTANS-LIKE, and TIMING OF CAB EXPRESSION1) motif, and in turn CO activates florigen FT in leaf phloem tissue [46]. GI mainly contributes to *CO* activation through the degradation of the transcriptional repressors for *CO* expression [9, 36] and can directly activate *FT* expression through its binding on FT chromatin [35]. The stability of CO protein is the key factor for LD-specific *FT* expression [47]. In LD conditions, *CO* mRNA accumulates in the light period to produce CO protein, whereas in SD conditions, *CO* mRNA accumulation overlaps with the longer dark period that limits CO protein amount; CO protein is degraded in the dark through ubiquitination by the activity of a RING-finger ubiquitin ligase, CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1), and subsequent proteasome attack [19, 30]. Thus, CO protein specifically accumulates in the evening of LD, and then activates *FT* transcription. The mechanism of *FT* activation by CO is assumed to involve the HEAM ACTIVATOR PROTEIN (HAP) complex and CCAAT-box DNA interaction [50]. HAP complex is a transcriptional regulator that binds the CCAAT-box DNA sequence motif to activate gene expression in eukaryotes. The CCT domain of Arabidopsis CO has structural similarity to the DNA binding domain of the

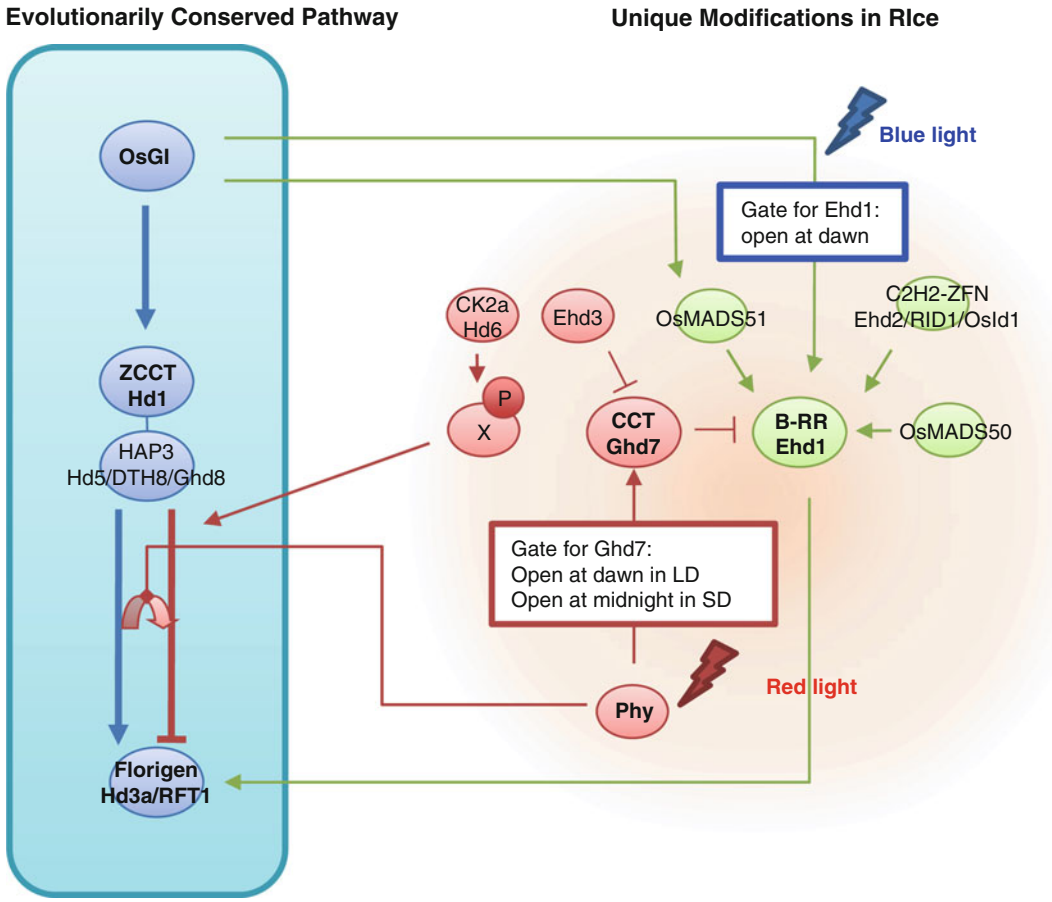

Fig. 18.2 Gene network for photoperiodic expression of florigen genes in rice. Evolutionarily conserved pathway that consists of the OsGI-Hd1-Hd3a/RFT1 establishes the basic mechanism for inducing flowering. In rice, evolutionarily unique modifications on the conserved pathways shape specific responses to the photoperiod. The key mechanism for regulation is the phytochrome-mediated conversion of Hd1 protein activity that promotes Hd3a expression in SD conditions but represses Hd3a expression in LD conditions. Ehd1 regulation through Ghd7 and several transcription factors also affect expression of florigen genes

HAP2 subunit and is required for the formation of the HAP2/HAP3/HAP5 tri-protein core of the HAP complex [50]. CO protein may directly induce *FT* expression, because when combined with HAP subunits CO can interact with the evolutionarily conserved CCAAT-box motif in the *FT* promoter in vitro [2, 4, 26, 43], and experiments using an inducible activation system indicate that CO can activate *FT* expression without newly translated proteins [54].

The rice counterpart of the GI-CO-FT pathway is composed of orthologous proteins, OsGIGANTEA (OsGI)-Heading date 1 (Hd1)-Hd3a, whereas the photoperiodic control of this pathway is completely reversed; the rice pathway is active specifically in SD conditions and modified to repress its activity in LD conditions [11]. *OsGI* was identified by the differential display approach [10], and transcriptome analysis using an *osgi* mutant revealed its fundamental role on the diurnal expression of clock-controlled genes, a function reminiscent of Arabidopsis GI [17]. Functional analyses using overexpression, RNAi, and *osgi* mutants also confirmed the conserved role of OsGI on *Hd1* activation [11, 17, 20]. It is not known whether this activation involves a system similar to Arabidopsis, such as the degradation of putative repressors for *Hd1* expression,

but the difference between *OsGI* expression in the evening and *Hd1* expression at midnight suggests an additional timing mechanism for *Hd1* activation [11].

Hd1 then activates *Hd3a* expression in SD conditions. This activation involves a unique adaptation to the photoperiodic response of rice, where different day lengths modulate different Hd1 activities. In contrast to Arabidopsis CO protein that is destabilized in the dark, rice Hd1 protein stability is not affected by the presence or absence of a light signal [12]. In addition, the role of COP1 in CO/Hd1 seems different in Arabidopsis and rice because the Arabidopsis *cop1* mutant accumulates more FT than WT does, whereas the rice *COP1* mutant, *peter pan syndrome* (*pps*), accumulates an extremely low level of *Hd3a* mRNA [41]. These differences make Hd1 the activator of Hd3a expression in SD conditions.

The most important control mechanism for photoperiodic florigen expression is that Hd1 activity is converted from an activator to a repressor of *Hd3a* expression in LD conditions. This conversion is mediated by the red light photoreceptor phytochrome B (phyB) [12, 18]. Three lines of evidences indicate this interesting modification on Hd1 action. First, near-isogenic lines containing *hd1* mutant alleles showed not only delayed flowering under SD conditions but also early flowering under LD conditions, indicating that Hd1 function is converted depending on day length [29]. This conversion is not observed in the phytochrome-deficient mutant *se5* (which lacks all phytochromes) [18]. Second, when *OsGI* is overexpressed, *Hd1* mRNA levels increase under both SD and LD conditions, whereas *Hd3a* mRNA levels are negatively correlated with *Hd1* transcript abundance, indicating that when highly induced *Hd1* can act as a suppressor of *Hd3a* [11]. Comparison of the expression patterns for *OsGI*, *Hd1*, and *Hd3a* suggested that *Hd1* expression in the presence of a light signal converts its activity to repress *Hd3a* expression. This hypothesis is directly confirmed by the combination of overexpression of *Hd1* and mutation of photoreceptors. *Hd1* overexpression delays flowering in SD conditions through suppression of *Hd3a* expression, probably because Hd1 accumulation in the light period changes its activity to function as a repressor even in the inductive SD conditions [12, 13]. This suppression is abolished in the *phytochrome B* mutants, clearly indicating that light signals mediated by phyB convert Hd1 function. In addition, the critical day-length response in which a short increment of day length greatly delays flowering is characterized by strong suppression of *Hd3a* expression by light, and this response is abolished in the *phyB* mutant. Third, a short exposure of light during the dark period disrupts the SD response in short-day plants including rice [14]. This phenomenon is referred to night break. Night break suppresses *Hd3a* expression at the transcriptional level, and night break experiments using *hd1* and *phy* mutants indicate that the night break signal mediated by phyB probably acts via the activities of Hd1 [14]. Altogether, the photoperiodic control of florigen expression through the OsGI-Hd1-Hd3a pathway is explained as follows: *Hd1* expression is regulated by the circadian clock component OsGI and peaks during darkness to accumulate Hd1 protein. Under SD conditions, Hd1 protein works as activator of *Hd3a* transcription because there is a weak effect on the light signal. Under LD conditions, *Hd1* expression partly overlaps with the end of the light period, generating a phyB-mediated light signal that converts Hd1 into the repressor. Night break under SD conditions is also mediated by the phyB signaling pathway and produces the LD-type repressive Hd1 to suppress *Hd3a* expression [12].

Several proteins that affect Hd1 activity have been reported. The HAP complex also regulates flowering time in rice. *Hd5*/*DTH8*/*Ghd8*, a QTL that delays flowering under LD conditions, encodes the HAP3 subunit. *Hd5*/*DTH8*/*Ghd8* requires a functional *Hd1* to delay flowering [7, 28, 49, 55]. In addition, overexpression of this gene activates *Hd3a* and *RFT1* expression and promotes flowering in SD conditions, but the inverse effect appears in LD conditions, suggesting that Hd1-HAP interaction similar to that in Arabidopsis contributes to flowering. *Hd1* mutant alleles lacking the CCT motif, which is sufficient

to interact with HAP proteins for Arabidopsis CO, have been identified from natural variants of cultivated rice, and these mutant alleles encode nonfunctional proteins [39].

The repressive activity of Hd1 is enhanced by casein kinase 2 (CK2) activity that includes Hd6 protein as the CK2α subunit [38]. When *Hd1* is functional, Hd6 CK2α clearly delays flowering and efficiently suppresses *Hd3a* expression in LD conditions, but these effects are abolished in SD conditions. Hd6 CK2α does not phosphorylate Hd1 protein directly. It is possible that an LD-specific protein complex that affects Hd1 repressor activity is the substrate for Hd6 CK2α [32].

7 Uniquely Acquired Pathway

Early heading date 1 (Ehd1), the B-type response regulator protein, is an important activator of *Hd3a* and *RFT1* expression [6]. *Ehd1* is an evolutionarily unique gene present in the rice (and other cereals) genome that constitutes a unique genetic network to control florigen regulation. The effect of *Ehd1* on flowering time is obvious in LD conditions in a normal (*Hd1* functional) background because in SD conditions the Hd1 pathway partially masks *Ehd1* function, but in LD conditions the Hd1 pathway turns into a repressor and Ehd1 becomes the sole activator for flowering [8].

Ehd1 expression is also controlled by a uniquely established gene network in rice. Activation of *Ehd1* involves three distinct regulatory units, *OsMADS50*, *Ehd2/Rice Indeterminate1* (*RID1*)/*Oryza sativa Indeterminate1* (*OsID1*), and *OsGI-OsMADS51*, all of which are unique to the rice genome. An important *Ehd1* activator is *OsMADS50*, a homolog of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) in Arabidopsis [27]. *SOC1* in Arabidopsis strongly promotes flowering downstream of FT in the shoot apex, whereas *OsMADS50* in rice activates *Ehd1*, an upstream regulator of *Hd3a* and *RFT1*, in the leaves [25]. These two genes act in the different organs in different contexts. Molecular genetic analysis using an *osmads50* mutant revealed the *OsMADS50-Ehd1-RFT1* pathway that promotes flowering in the noninductive LD conditions.

Ehd2/Rice Indeterminate1 (*RID1*)/*Oryza sativa Indeterminate1* (*OsID1*), an ortholog of maize *indeterminate1* (*id1*) that encodes a C2-H2 zinc finger protein, is also required for *Ehd1* expression [31, 34, 52].

Mutation in the *Ehd2/RID1/OsId1* gene reduces the transcript accumulation of both *Ehd1* and *Hd1* but has no effect on other regulators such as *OsMADS50* or *OsGI*. This result suggests an interplay between the conserved *OsGI-Hd1-Hd3a* pathway and the unique *Ehd1* pathways through *Ehd2*. In this context, OsGI works as an activator for both *Hd1* and *Ehd1*, the latter through the up-regulation of *OsMADS51*, a transcription factor specifically found in the rice genome [20]. Genetic analyses indicate that the *OsGI-OsMADS51* pathway activates *Ehd1* specifically in SD conditions but has no effect on *Hd1* expression.

Recently, a novel function of *OsGI* was revealed that acts through blue light signaling which activates *Ehd1* expression at the beginning of a light period [15]. The blue light strongly activates *Ehd1* expression when illuminated in the morning. This blue light response requires *OsGI*.

The above-mentioned pathways are all the activators for *Ehd1* expression. The repressor of *Ehd1* expression, *Ghd7* (for *Grain number, plant height, and heading date 7*), also plays important roles in the photoperiodic control [53], and detailed physiological analyses revealed that an interaction between blue light-*Ehd1* signaling and red light-*Ghd7* signaling contributes to the setting of the critical day-length response in photoperiodic florigen expression [15]. *Ghd7* was first identified from QTLs affecting pleiotropic agronomic traits including flowering. Functional *Ghd7* represses *Ehd1* and downstream *Hd3a/RFT1* expression in LD conditions. *Ghd7* is up-regulated by red light signaling mediated by phytochromes [33], and interestingly, the effect of red light is limited to midnight in SD conditions and in the morning in LD conditions. This result means that the window of time in which phytochrome signaling is allowed to transduce is changed by day length. In SD conditions, *Ghd7* is

not induced by red light because the photo-sensitive phase is at midnight. In LD conditions, the photo-sensitive phase moves to the morning, and *Ghd7* is immediately up-regulated at dawn to suppress *Ehd1* and subsequent *Hd3a* expression [15].

8 Natural Variations in Flowering

Modern rice cultivars have been established through about 8,000 years of domestication and breeding, and these efforts enabled the spread of cultivation areas beyond the original area of domestication. This expansion was possible due to natural variation in genes involved in the conserved and unique flowering pathways [16]. The expression level of *Hd3a* is highly correlated with flowering time in SD conditions among the rice cultivars collected worldwide [39]. This variation in *Hd3a* expression is largely explained by the high degree of polymorphism in the *Hd1* gene, where functional and nonfunctional alleles are associated with early and late flowering. Interestingly, nonfunctional alleles of *Hd1* were rarely found in *Oryza ruffipogon*, the wild ancestor of cultivated rice *Oryza sativa*, suggesting that selection of nonfunctional *Hd1* genes contributed to the domestication of modern cultivars [37].

Although the *Ehd1* gene does not contain any polymorphisms that result in loss of function except for an old Taiwan variety, expression of the *Ehd1* gene was highly correlated with *Hd3a* expression levels, suggesting that the regulator of *Ehd1* expression could contribute to the variation in flowering time [39]. *Ghd7*, which suppresses *Ehd1* expression, shows substantial polymorphism linked with flowering time [7, 53]. A strong allele of *Ghd7* tends to be found in the southern part of Asia, but weaker or nonfunctional alleles appear in the northern part of Asia where early flowering cultivars are preferred. Extended QTL analyses also revealed the genetic architecture of flowering in rice. Major genetic factors consisting of *Hd1*, *Hd3a*, *Hd6*, *RFT1*, and *Ghd7* affect flowering time in a Japanese cultivar and diverged Asian cultivars [7].

9 Conclusion

Recent molecular genetic studies in rice represent a substantial advance in our understanding of the molecular action of florigen, control of florigen expression through conserved and unique pathways, and the molecular basis for natural variation in flowering time. These studies provide the molecular tools for controlling the flowering of rice and other economically important plants such as biofuel cereals.

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Panicle Development

19

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1 Introduction

Rice reproductive development plays an essential role in determining grain yield and includes the formation of male and female organs supported by the associated structures including inflorescences and spikelets. Rice is a monoecious plant; its inflorescence is determinate and has around ten or more lateral branches (called primary branches) (Fig. 19.1a–c). Condensed spikes (also called spikelets) are directly initiated on the primary and secondary branches (Fig. 19.1c). Spikelets are arranged on the primary branches in a biased distichous phyllotaxy [1]. Rice spikelet development initiates with the formation of a pair of rudimentary glume primordia and terminates after the formation of the carpel (Fig. 19.1d) [2, 3]. A rice spikelet consists of two pairs of repressed bracts, i.e., rudimentary glumes and sterile lemmas (or called empty glumes), and a single fertile floret that contains a lemma and a palea in whorl 1, two lodicules in whorl 2, six stamens in whorl 3, and a pistil in whorl 4 [2, 3] (Fig. 19.1e). In this chapter, we summarize the recent understanding of genetic mechanism in specifying rice reproductive development.

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2 Regulation of Meristem Size of Inflorescence and Spikelet

The central region in the plant aerial meristem contains a group of pluripotent and undifferentiated cells (also called stem cells) which have the ability to self-maintain and differentiate into daughter cells to form leaf and floral organ primordia in the peripheral zone. The developmental switch of the shoot apical meristem from vegetative to reproductive meristem and the subsequent inflorescence and flower development are tightly regulated by both environmental and endogenous signals.

Rice flowering time genes, including *Heading date 1* (*HD1*) *OsMADS51*, *Early heading date 1* (*EHD1*), *Hd3a,* and *Flowering-locus T 1* (*RFT1*), promote floral transition under short day (SD) conditions and the formation of reproductive meristems and play roles in determining the panicle shape and branch and grain numbers [4–11]. Under long day (LD), *HD1* turn to be an inhibitor, and additional repressor genes, *Ghd7* and *OsMADS50*, control flowering time by repressing the transcription of *EHD1* [12–15].

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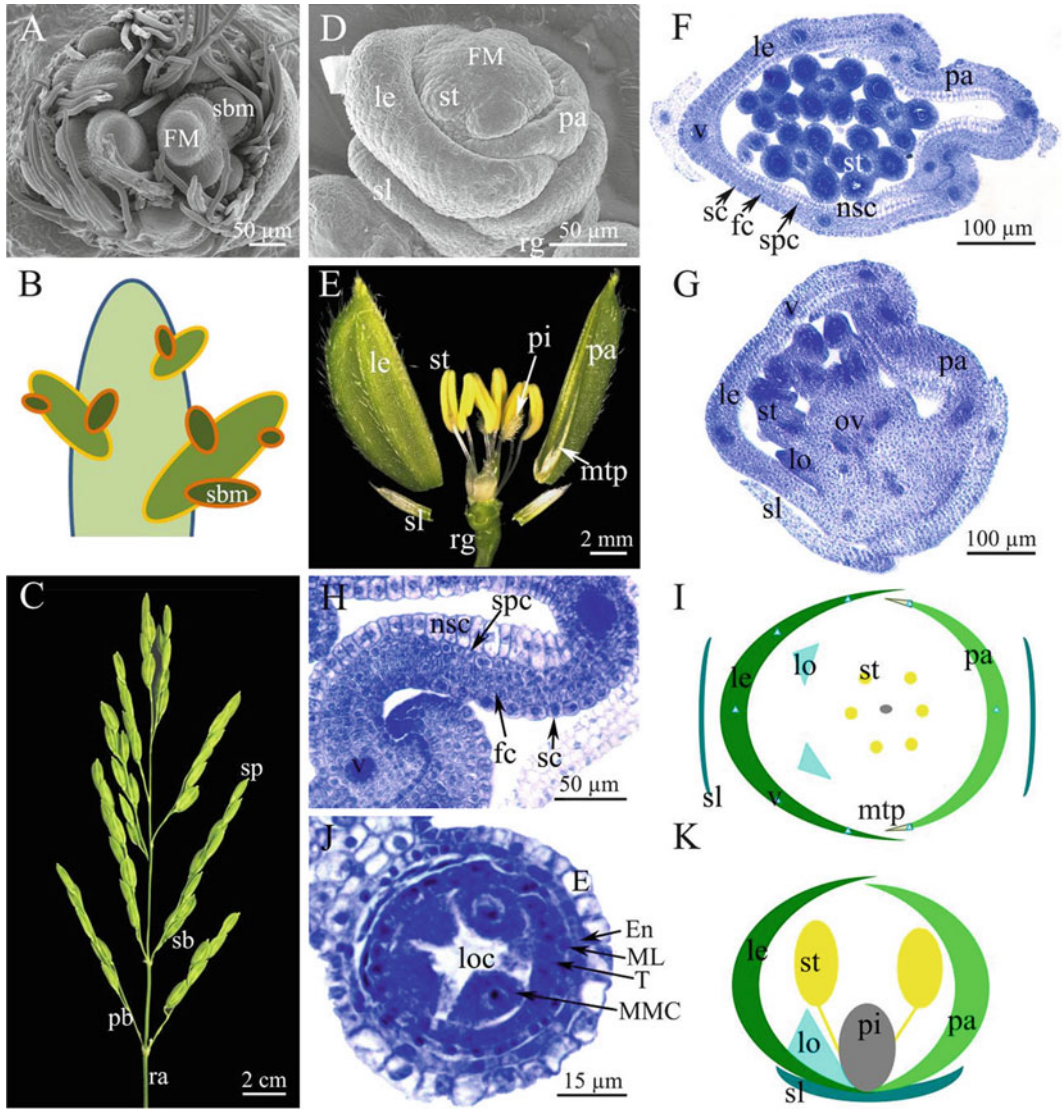

Fig. 19.1 Rice inflorescence and spikelet development. (**a**, **b**): SEM (**a**) and diagram (**b**) of inflorescence meristem showing secondary branch meristem (*sbm*) and floral meristem (*FM*). (**c**) Rice inflorescence showing the inflorescence axis, and spikelets (*sp*) attached to the primary branch (*pb*) and secondary branches (*sb*). (**d**) SEM of the rice spikelet meristem showing primordia of the rudimentary glume (*rg*), sterile lemma (*sl*), lemma (*le*), palea (*pa*), and stamen (*st*). (**e**) Rice spikelet showing rudimentary glumes, sterile lemmas, the lemma, the palea, stamens, and the pistil. (**f**, **g**) Transverse (**f**) and longitudinal (**g**) paraffin sections of a rice spikelet. (**h**) A close-up of the lemma in (**f**) showing silicified cells (*sc*), fibrous sclerenchyma (*fs*), spongy parenchymatous cells (*spc*), and non-silicified cells (*nsc*). (**j**) Paraffin section of an anther showing locule (*loc*), epidermis (*e*), endothecium (*En*), middle layer (*ML*), tapetum (*T*), and microspore mother cell (*MMC*). (**i**, **k**) Diagrams of flowers from transverse (**f**) and longitudinal (**g**) sections. *E* epidermis, *En* endothecium, *FM* flower meristem, *le* lemma, *MC* meiotic cell, *ML* middle layer, *mtp* margin tissue of palea, *lo* lodicules, *loc* locule, *pa* palea, *pi* pistil, *rg* rudimentary glume, *nsc* non-silicified cell, *sc* silicified cell, *sg* sterile glume, *spc* spongy parenchymatous cell, *st* stamen, *T* tapetum, *v* vascular bundle

The activity and differentiation of the stem cells are tightly controlled in plants. One of the best characterized shoot apical meristem signaling pathways in *Arabidopsis* is the *CLAVATA* (*CLV*) pathway, which controls the stem cell niche by the function of at least three genes, *CLV1-CLV3*. Recently, increasing evidence indicates that the CLV signaling pathway is functionally

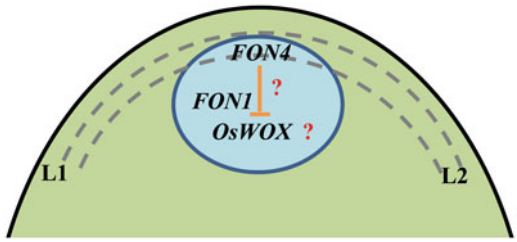

Fig. 19.2 Working model for rice inflorescence and spikelet meristem maintenance. See text in the section of "Regulation of Meristem Size of Reproductive Structures" for details of the model. *FON1 Floral Organ Number 1*, *FON4 Floral Organ Number 4*, *L1* epidermal layer from the outer layers, *OsWOX WUSCHEL-related homeobox* gene family in rice

conserved in monocots and eudicots [3, 16, 17]. The rice *floral organ number 1* (*fon1*) mutant displays an enlarged floral meristem and an increased number of all floral organs; for example, the stamen number in *fon1-2* is increased to 7-12 [18]. *FON1* encodes a protein orthologous to CLV1 [19]. Furthermore, the rice mutants *floral organ number 4* (*fon4*) were reported to exhibit enlargement of the shoot apical meristem, inflorescence meristem, and floral meristem, and as a result, an increase in the number of all floral organs, particularly the inner floral organs [20]. This defect is very similar to that of *fon1*. *FON4* (also called *FON2*) encodes a putative secreted peptide orthologous to CLV3 and is mainly expressed in small groups of cells at the apex of the vegetative shoot apical meristem, the inflorescence meristem, and the floral meristem [20–22] (Fig. 19.2). In the rice genome, there are 13 putative *WOX* (*WUSCHEL-related homeobox* gene family) genes, among which *OsWUS* is closely related in sequence to the *Arabidopsis WUS* gene [23–25]. However, the biological function of *OsWUS* remains unknown. *OsWUS* is not expressed at the organizing center of the vegetative shoot apical meristem as the *Arabidopsis WUS* gene [25] (Fig. 19.2). Instead, the expression of grass *WUS* homologs is in L1 layer of branch meristems during the early stages, and later uniformly through the meristem, which are consistent with the expression patterns of *FON1*, implying the divergent role of *WUS* homologs in stem cell homeostasis in grasses [25].

Hormones such as auxin and cytokinin play a role in regulating meristem initiation and the size of inflorescences and flowers in grasses, with auxin involved in axillary meristem initiation and cytokinin regulating meristem size [26, 27]. *LOG* (*Lonely Guy*) is a rice gene specifically expressed at the top of shoot, inflorescence, and floral meristems and encoding a novel enzyme with phosphoribohydrolase activity, which can directly convert inactive cytokinin nucleotides to the bioactive form [27]. A major rice quantitative trait locus (QTL), *GRAIN NUMBER1* (*GN1*), encodes a cytokinin oxidase (CKX2) that degrades cytokinin. Reduced expression level of *GN1* results in an increase of cytokinin levels in inflorescence meristems and a higher number of branches and spikelets in inflorescence [28] (Fig. 19.3).

3 Control of Inflorescence Development

In rice, branched inflorescence structure and spikelet organs contribute to its architecture [29]. Genome-wide transcriptome profiling using the inflorescence/panicle primordia from phase transition to floral organ differentiation revealed that 357 out of 22,000 genes are differentially expressed, suggesting the vast number of genes involved in controlling rice inflorescence development [30]. Additionally, small regulatory RNAs may play a role in early inflorescence development rice. Johnson and his coworkers observed that 831 small RNA clusters, which primarily contain 21-nt small RNAs and phased RNAs, were preferentially expressed in developing inflorescences; furthermore, 38 of the 24-mer clusters were phased and preferentially expressed in inflorescences [31].

Activity of axillary meristems plays an essential role in determining the plant architecture during both vegetative and reproductive stages. In *Arabidopsis*, the transcription factor LEAFY (LFY) promotes the vegetative-to-reproductive transition and specifies floral meristems, which arise from the periphery of the reproductive shoot apex. Rice *LEAFY* (*RFL*) has a distinctive expression pattern from that of any other *LFY*-like

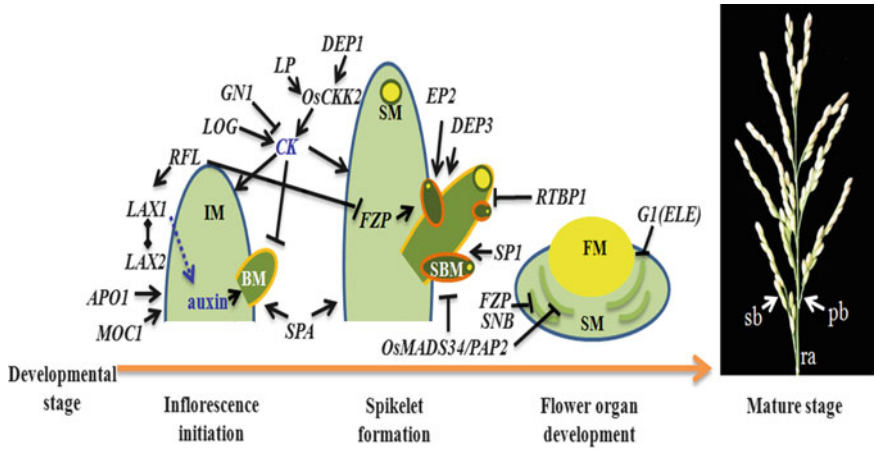

Fig. 19.3 Molecular control for rice inflorescence and spikelet development. See text in the section of "Control of Inflorescence Development for details of the model." *APO1 Aberrant Panicle Organization 1*, *BM* branch meristem, *CK* cytokinin, *DEP DENSE AND ERECT PANICLE*, *DEP3 DENSE AND ERECT PANICLE 3*, *EP2 Erect Panicle 2*, *FM* floral meristem, *FZP* Frizzy Panicle, *G1/ELE Long Sterile Lemma 1/Elongated Empty Glume*, *GN1 GRAIN NUMBER1*, *IM* inflorescence meristem, *MOC1 MONOCULM 1*, *LAX1 LAX PANICLE 1*, *LAX2 LAX PANICLE 2*, *LOG Lonely Guy*, *PAP2 PANICLE PHYTOMER 2*, *RFL Rice LEAFY like* gene, *RTBP1 RICE TELOMERE BINDING PROTEIN1*, *SBM* secondary branch meristem, *SM* spikelet meristem, *SNB Supernumerary Bract*, *SPA Small Panicle*, *SP1 SHORT PANICLE1*

genes in *Arabidopsis* and *Antirrhinum majus*. *RFL* is highly expressed in axillary meristems, including tiller buds, and in the apical regions of inflorescence and branch meristems. Knockdown of *RFL* causes a reduction of the number of tillers and inflorescence branches, suggesting that RFL is a key regulator of vegetative to inflorescence meristem transition as well as of inflorescence shaping in rice [32]. *RFL* is the earlier switch for inflorescence development, because its expression precedes that of either *LAX PANICLE1* (*LAX1*) or *Frizzy Panicle* (*FZP*). RT-PCR analysis revealed a down-regulation of *LAX1* and an up-regulation of *FZP* in *RFL* RNA interference lines, suggesting that RFL may determine rice plant architecture via regulating distinct pathways [32] (Fig. 19.3).

The *FZP*/*BRANCHED FLORETLESS 1* (*BFL1*) gene was shown to be required for suppressing the formation of axillary meristems within the spikelet meristem and to promote the subsequent specification of floral meristem identity. The *FZP* gene encodes an ethylene-responsive element-binding factor (ERF) and is expressed at the axils of the rudimentary glume primordia [33–35] (Fig. 19.3). *LAX1* encodes a basic helix-loop-helix (bHLH) transcription factor expressed in the boundary between the shoot apical meristem and the region of new meristem formation. LAX1 regulates the axillary meristem generation during rice reproductive development (Fig. 19.3). Genetically, *LAX1* plays an overlapping role with another panicle branching regulator, Small Panicle (SPA), in axillary meristem initiation [33, 34] (Fig. 19.3). LAX2 is another key regulator in rice axillary meristem determination. *LAX2* encodes a novel nuclear protein, which has a plant-specific conserved domain and is able to physically interact with LAX1 [36].

The phytohormone auxin is essential for the initiation of axillary meristems and the specification of secondary branching [37–39]. Auxin maximum forms at the place of axillary meristem initiation and is consumed when the primordia growth occurs, followed by the formation of a new auxin sink where the next primordium is generated [40–42]. *LAX1* and its maize homolog *BARREN STALK1* (*BA1*) have been found to regulate auxin biosynthesis or transport during inflorescence development [41, 43] (Fig. 19.3).

Furthermore, Oikawa and Kyozuka demonstrated that outgrowth of the axillary meristem only occurs when the LAX protein is trafficked into the site of primordium initiation [43].

MONOCULM 1 (*MOC1*) encodes a GRAS family nuclear protein putatively orthologous to the *Arabidopsis* LATERAL SUPPRESSOR (LAS) [44, 45]. *MOC1* is expressed mainly in the axillary buds and is required for the initiation of axillary buds and inflorescence branch meristem (Fig. 19.3). Because of a failure in the initiation of axillary meristems, the *moc1* mutant plants have no tillers and panicles but only a main culm with much fewer branches and spikelets [45].

Rice *Aberrant Panicle Organization 1* (*APO1*) is the ortholog of *FIMBRIATA* (*FIM*) in *Anthirrhinum* and *UNUSUAL FLORAL ORGAN* (*UFO*) of *Arabidopsis*, which encodes an F-box protein that regulates meristem fate [46, 47]. Rice *APO1* was shown to positively regulate the number of primary branches and spikelets (Fig. 19.3) [48, 49]. The erect panicle phenotype in some rice varieties is of importance for yield, because of the higher photosynthetic capacity of these tissues [50]. Recently, a QTL responsible for erect panicle and high grain number per panicle traits, *DENSE AND ERECT PANICLE1* (*DEP1*), also called *qPE9-1*, was identified on chromosome 9, and this erect panicle phenotype is due to the enhanced meristematic activity and cell proliferation through regulating OsCKX2 [51–54]. *DEP1* encodes an unknown protein that contains the PEBP (phosphatidylethanolamine-binding protein) domain, which shares some similarity with the N terminus of GS3 [51] (Fig. 19.3). In addition, the *ep2* (*erect panicle 2*) mutants have erect panicles, altered panicle length and grain size, and have more vascular bundles and a thicker stem compared with wild-type plants. *EP2* is specifically expressed in the vascular bundles of internodes and encodes a novel plant-specific protein with unknown biochemical function, which is localized in the endoplasmic reticulum [55] (Fig. 19.3). Furthermore, DEP3, a patatin-like phospholipase A2 (PLA2) superfamily domain-containing protein [56], was identified as another regulator of rice inflorescence development (Fig. 19.3).

Molecular and genetic evidence on model eudicot plants such as *Arabidopsis* and *A. majus* led to the classic genetic ABC and revised ABCE models to explain the specification of floral organ identity [57–59]. E-class genes *SEPALLATA* (*SEP*) act as integrating co-factors with other floral identity genes in determining floral organ identity [60]. Grasses have diverse *SEP*-like genes, and at least five of them (*OsMADS1*, *OsMADS5*, *OsMADS7*, *OsMADS8*, and *OsMADS34*) are found in rice [61–64]. It has been recently shown that one of these rice *SEP-like* genes, *OsMADS34* (also called *PANICLE PHYTOMER2*, *PAP2*), is involved in controlling rice inflorescence and spikelet morphology by determining the number of branches and spikelets and in the specification of the sterile lemmas [65, 66] (Fig. 19.3).

LARGER PANICLE (*LP*) encodes a Kelch repeat-containing F-box protein and is mainly expressed in the branch primordia (Fig. 19.3). LP is localized to the endoplasmic reticulum (ER) and is able to interact with a rice SKP1-like protein, suggesting that LP may be involved in ER-associated protein degradation [67]. *SHORT PANICLE1* (*SP1*) encodes a putative peptide transporter, which contains a conserved PTR2 domain with 12 transmembrane domains, and is highly expressed in the phloem of the branches of young panicles. *SP1* was shown to positively regulate rice panicle elongation (Fig. 19.3), and *sp1* develops a short-panicle phenotype [68]. *RICE TELOMERE BINDING PROTEIN1* (*RTBP1*) encodes a telomere binding protein, which affects both germination and postgermination growth of vegetative organs over four generations, as well as reproductive organ formation [69] (Fig. 19.3).

4 Spikelet Organ Development

A rice spikelet has two pairs of rudimentary glumes and sterile lemmas, and a single fertile floret containing a lemma and a palea, two lodicules, six stamens, and a pistil (Fig. 19.1). The two rudimentary glumes in rice bear no axillary buds. The sterile lemmas are much larger than the

rudimentary glumes yet much smaller than the lemma and the palea. It is hypothesized that the leaf-like structures, lemma and palea, are homologous to the *Arabidopsis* sepal and the fleshly or scale-like lodicules are homologous to the *Arabidopsis* petals [70, 71]. Two rudimentary glumes, two sterile lemmas, and lemma and palea are organized in 1/2 alternate phyllotaxy in a rice spikelet [1]. A rice floret has a bilateral symmetric pattern along the lemma-to-palea (Le/Pa) axis and consists of a lemma, a palea, and lodicules based on their unique position and morphology (Fig. 19.1f, i) [2]. The outer whorl and the second whorl in the rice floret are not radially symmetrical, i.e., the lemma and palea develop interlocking structures, the lemma is larger than the palea, and the second whorl consists of two lodicules close to the lemma (Fig. 19.1e, f, i). However, the six stamens in each rice floret are symmetrically located in the third whorl around the pistil, which is similar to the classic symmetrical arrangement of floral organs in eudicots. After the formation of floral organs, the floral meristem is terminated by the formation of an ovule primordium inside the pistil (Fig. 19.1e, g).

4.1 Development of Rudimentary Glumes and Sterile Lemmas

Each rice spikelet contains two highly reduced leaf-like rudimentary glumes at the base in a distichous pattern and two reduced sterile lemmas at a position opposite to each other above the rudimentary glumes [1]. Compared with lemma and palea, sterile lemmas are smaller in size and exhibit characteristic cellular patterns [72]. It has been hypothesized that sterile lemmas are vestiges derived from lemmas of two lateral sterile florets during evolution [73, 74]. Recent evidence suggests that *G1* (*Long Sterile Lemma1*)/*ELE* (*Elongated Empty Glume*) and *OsMADS34* play key roles in repressing lemma identity at the sterile lemma positions during rice spikelet development (Fig. 19.4) [65, 75, 76]. *G1*/*ELE* encodes a plant-specific protein with a domain of unknown function, ALOG (for *Arabidopsis* LIGHT-DEPENDENT SHORT HYPOCOTYLS1 and *Oryza* G1) [75, 76]. *G1*/*ELE* and *OsMADS34* are expressed in sterile lemma primordia throughout development.

FZP and *SNB* (*Supernumerary Bract*) were shown to regulate the development of rudimentary glumes (Fig. 19.4) [33, 77]. Rice *SNB* and *OsIDS1* encode proteins with AP2 domains [77, 78]. *SNB* is required for the transition from spikelet meristem to floral meristem and floral organ development and is strongly expressed in newly emerging spikelet meristems. Similar to what was found in *Arabidopsis*, the expression of *SNB* and other rice AP2 family gene(s) is repressed by miR172 [79].

4.2 Specification of Lemma/Palea and the Uncertain Role of the A-class Genes

Rice contains at least 75 MADS box genes, some of which have significant similarity to the *Arabidopsis* ABCDE genes [64]. Recent emerging evidence supports that rice has conserved and diversified floral developmental mechanism compared with eudicot model plants [3, 5, 17, 80–85].

Palea and lemma are unique structures observed only in the grasses, where they protect the florets and kernels from pathogen and insect attack besides supplying carbohydrates to the developing seeds [86]. As the outer floral organ, the lemma and palea form interlocking structures and have a very similar morphology, i.e., both of them contain silicified cells (sc), fibrous sclerenchyma (fs), spongy parenchymatous cells (spc), and non-silicified cells (nsc). However, the lemma and palea have distinctive cellular patterns [2, 91] (Fig. 19.1f). The palea contains a unique marginal tissue, which is absent in the lemma. This marginal tissue of the palea (mtp) is distinctive from the rest of the palea by having a unique and smooth epidermis that lacks epicuticular and silicified thickening (Fig. 19.1f). The second histological difference among the lemma, palea, and lodicules is that they have varied vascular tissue patterns, i.e., the lemma contains five vascular bundles, the palea has three, whereas in lodicules, several vascular trachea elements are distributed in the parenchyma cells [87, 88] (Fig. 19.1f).

Rice genome has four putative A class genes that encode *AP1*/*FRUITFULL* (*FUL*)-like proteins:

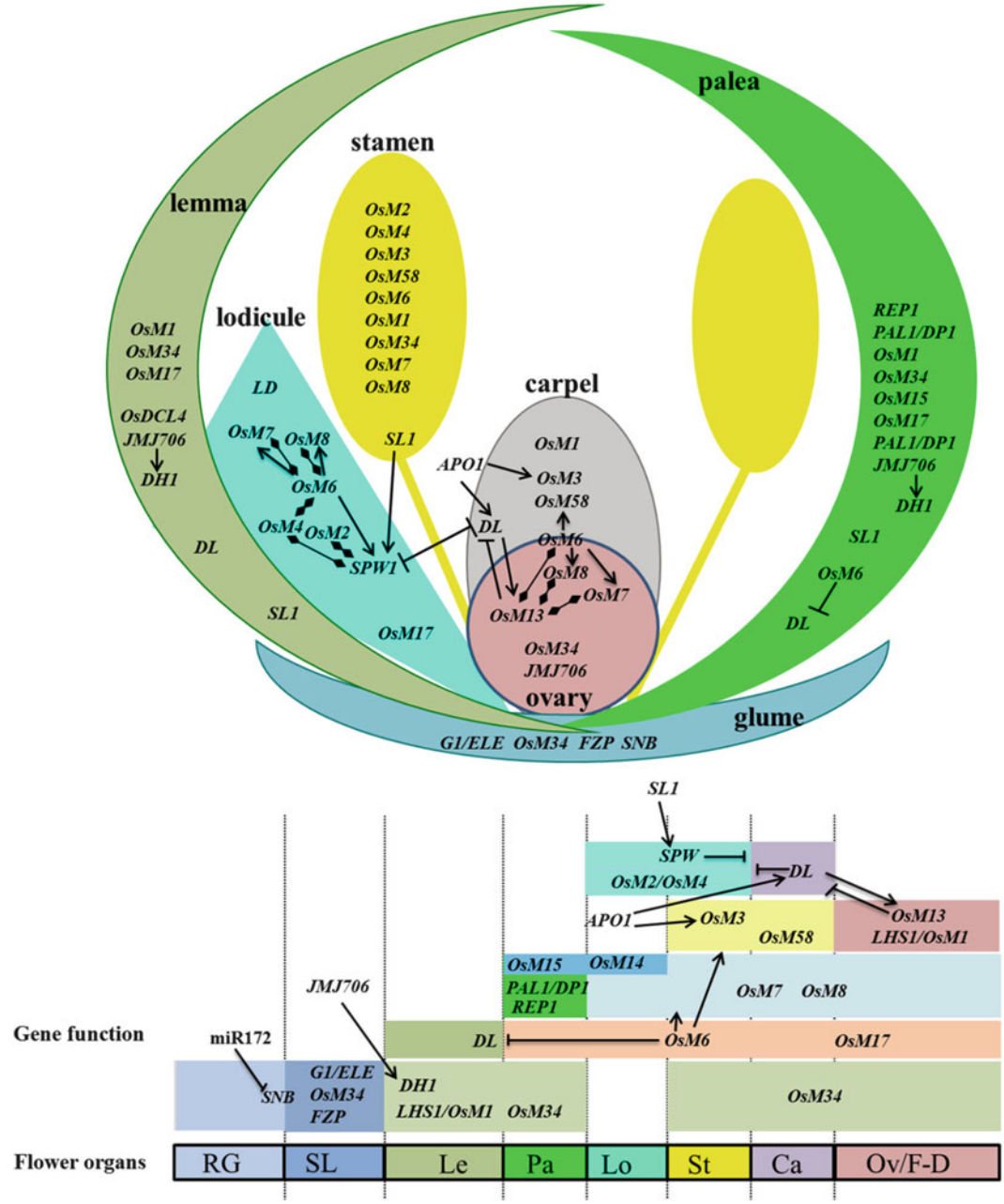

Fig. 19.4 Model for spikelet organ development. See text in the section of "Spikelet Organ Development" for details of the model. *Ca* carpel, *DH1 Degenerated Hull 1*, *DL DROOPING LEAF*, *F-D* flower determinacy, *FZP Frizzy Panicle*, *G1/ELE Long Sterile Lemma 1/Elongated Empty Glume*, *Le* lemma, *LHS1 Leafy Hull Sterile 1*, Lo lodicule, *OsM* is an abbreviation of *OsMADS*, *Ov* ovule, *Pa* palea, *PAL1/DP1 Paleless 1/DEPRESSED PALEA1*, *REP1 RETARDED PALEA1*, RG rudimentary glume, *SL* sterile lemma, *SL1 STAMENLESS 1*, *SNB Supernumerary Bract*, *St* stamen

OsMADS14, *OsMADS15* (also called *Degenerative Palea*, *DEP*), *OsMADS18*, and *OsMADS20* [81, 82]. However, the roles of class A genes in floral organ identity are not as clear as that was hypothesized by the ABDCE model because of the lack of mutants [82]. The *dep/osmads15* mutant displayed shrunken paleas and slightly elongated lemmas and glumes (Fig. 19.4) [89]. These phenotypes are

different from that of the mutants from the *Arabidopsis* class A genes *AP1* and *AP2*, which display the conversion of sepals into leaf- or bract-like structures and petals into stamen-like organs or loss of sepals [90, 91]. A recent study indicated that *PAP2*/*OsMADS34* interacts with three *APETALA1* (*AP1*)/*FRUITFULL* (*FUL*)-like genes (*OsMADS14*, *OsMADS15*, and *OsMADS18*) in the meristem to specify the identity of the inflorescence meristem downstream of the florigen signal. In the quadruple knockdown lines, the meristem continued to generate leaves, rather than becoming an inflorescence meristem [92]. Therefore, rice *AP1*/*FUL*-like genes seem functional similarity in promoting rice flower development as that of the *Arabidopsis* A-class genes. *AP2* transcription factors in rice have been shown to regulate shoot apical meristem determinacy. Mutations in the *AP2*-like gene *SNB* in rice result in delayed transition of shoot spikelet meristem to floral meristem, which produces additional bract-like organs [77].

OsMADS1/*LHS1* (*leafy hull sterile 1*), *OsMADS6*, *DROOPING LEAF* (*DL*), *RETARDED PALEA1* (*REP1*), and *DEPRESSED PALEA1* (*DP1*) are required for rice lemma/palea specification (Fig. 19.4). *LHS1* is an E-class gene that is able to specify the identity of lemma and palea and the identity of the inner floral meristem [88, 93–95]. Further, *LHS1* determines the lemma/palea identity together with *OsMADS34* [65]. *OsMADS6* belongs to the *AGAMOUS-LIKE6* (*AGL6*) MADS-box gene family, which is widely distributed in both gymnosperms and angiosperms. *OsMADS6* plays a key role in specifying floral organ and meristem identities in rice [96, 97] (Fig. 19.4). Another rice floral homeotic gene, *DL*, which encodes a *YABBY* transcription factor, is distinct from the well-known ABC genes in its sequence and function. The *dl* mutants exhibit the homeotic transformation of carpels into stamens and altered identity of the lemma, which contains three to five vascular tissues [98, 99]. Genetic and expression analyses suggest that both *OsMADS6* and *DL* are required for the proper establishment of palea identity and morphology and that *OsMADS6* specifies palea identity by repressing the expression of *DL* [99–101] (Fig. 19.4). *REP1* is a *CYCLOIDEA*-like gene in rice and controls palea identity and initiation by regulating cell proliferation and expansion, but it does not affect lemma development [2]. *DP1* encodes a nuclear-localized AT-hook DNA binding protein, and *dp1* mutants show a primary defect in the main structure of palea which is replaced by two leaf-like structures with the characteristics of mtp [100, 101] (Fig. 19.4).

4.3 Conserved and Diversified BCDE Genes

B-class genes have conserved functions in grasses. For example, mutations in rice *SUPERWOMAN1* (*SPW1*) (also called *OsMADS16*) (Fig. 19.4), which is the ortholog of the *Arabidopsis* B-function gene, *AP3*, result in homeotic transformations of stamens to carpels and lodicules to lemma- or palea-like structures [102–104]. Rice has two duplicated *PI* like genes, *OsMADS2* and *OsMADS4* (Fig. 19.4), and they were shown to redundantly regulate lodicule and stamen development [105, 106]. The rice *STAMENLESS 1* (*SL1*) gene encodes a C2H2 zinc finger protein, which is closely related to the *Arabidopsis* JAGGED (JAG) protein; the *sl1* mutants display homeotic conversions of lodicules and stamens to palea/lemma-like organs and carpel-like structures, phenotypes that are similar to that of *spw1*. These observations suggest the diversification of *JAG* and *SL1* during evolution. In addition, *SL1* is capable of activating the expression of *SPW1* [107] (Fig. 19.4).

Grasses contain duplicated and subfunctionalized C-class genes [108, 109], which retain conserved and diversified function compared with the eudicot C-class genes. In rice, there are two *AG* homologs, *OsMADS3* and *OsMADS58* [108]. *OsMADS3* has been shown to play a key role in stamen and ovule identity specification, late anther development, and floral meristem determinacy. *OsMADS58* plays a redundant role with *OsMADS3* in specifying the floral meristem determinacy and the identity of male and female reproductive organs [98, 110–112] (Fig. 19.4).

Rice contains two D-class genes, *OsMADS13* and *OsMADS21*, which are orthologous to *SEEDSTICK* (*STK*) in *Arabidopsis* and to *FLORAL BINDING PROTEIN7* (*FBP7*) and *FBP11* in petunia (*Petunia hybrida*). *OsMADS13* plays a key role in carpel/ovule identity specification and floral meristem termination [98, 113, 114] (Fig. 19.4). Unlike *OsMADS13*, the function of *OsMADS21* in flower development may have been lost during evolution [113]. Moreover, *OsMADS13* may act in the same pathway with *DL* in specifying the identity of carpel/ovule and floral meristem, because the defects of *osmads13 dl* flowers appeared identical to those of the *dl* mutant, and *OsMADS13* expression was undetectable in *dl* flowers [98] (Fig. 19.4).

OsMADS3, *OsMADS58*, *OsMADS13*, and *OsMADS21* belong to the AG subfamily of MADS-box proteins [108, 110, 113]. Genetic analyses indicate that Os*MADS3* and *OsMADS13* have retained their conserved function in carpel/ovule development and floral meristem determinacy even though they had undergone multiple subfunctionalization and/or neofunctionalization events after duplication [98, 110] (Fig. 19.4).

Grasses have E-class genes that are diversified in sequence and function [62–64, 98]. *LHS1* was shown to specify the identity of lemma and palea and the identity of the inner floral meristem [88, 93–95, 115]. Transgenic plants with reduced expression of both *OsMADS7* and *OsMADS8* exhibit late flowering, homeotic transformations of lodicules, stamens and carpels into palea/lemma-like organs, and a loss of floral determinacy. Simultaneous reduction of the expression of four rice *SEP*-like genes, i.e., *LHS1*, *OsMADS5*, *OsMADS7,* and *OsMADS8*, causes homeotic transformation of all floral organs except the lemma into leaf-like organs [116] (Fig. 19.4). *OsMADS34* (*PAP2*) controls the development of inflorescences and spikelets [65, 66]. Analysis of os*mads34 osmads1* suggests that *OsMADS34* and *LHS1* redundantly specify the identities of floral organs, including lemma/palea, lodicules, stamens, and carpel [65] (Fig. 19.4).

4.4 AGL6 Genes Possess the E-class Function

Phylogenetic analyses indicated that the *AGAMOUS-LIKE6* (*AGL6*)-like genes have high sequence similarities, and form sister clades on the phylogenic tree, to the *SEP*-like genes [63, 96, 117, 118]. *SEP*-like genes are only found in angiosperms, while *AGL6* genes are widely distributed in gymnosperms and angiosperms. Recent evidence indicates that *AGL6*-like genes in monocots and eudicots have *SEP*-like functions in flower development [96, 97, 119–121] (Fig. 19.4). Rice contains two *AGL6* members: *OsMADS6* and *OsMADS17*. *OsMADS6* is highly expressed in floral meristem at early stages and in the palea and inner floral organ primordia (lodicule and pistil) at later stages [96, 97, 119]. Genetic analyses suggest that *OsMADS6* specifies the identity of the three inner whorls and floral meristem determinacy redundantly with *SPW1* (B-gene) or *OsMADS3* (C-gene). In addition, *OsMADS6* defines carpel/ovule identity and floral determinacy by interacting with *OsMADS13* (D-gene) and specifies the palea and floral meristem identities together with *DL* (Fig. 19.4). Moreover, *OsMADS6* can activate the expression of B-, C-, and E-class genes at the early flower developmental stage at the transcription level (Fig. 19.4) [98]. Lastly, *OsMADS6* is able to specify the "floral state" by determining floral organ and meristem identities together with *LHS1*/*OsMADS1*, as revealed by the analysis of double mutants. Indeed, the *osmads1 osmads6* double mutants display severe floral defects such as no inner floral organs or glume-like structures within flowers and strongly indeterminate floral meristem [96, 97].

4.5 Epigenetic Control of Spikelet Development

Increasing evidence suggests that epigenetic control is essential for rice spikelet development. Small noncoding RNA molecules including MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) play fundamental roles in development, epigenetic

modifications, and viral defense. These small regulatory RNAs are derived from different types of precursors and processed by distinct Dicer or Dicer-like (DCL) proteins. Rice DCL4 was shown to be the major Dicer responsible for the 21-nucleotide siRNAs associated with inverted repeat transgenes and for the trans-acting siRNA (ta-siRNA) from the endogenous TRANS-ACTING siRNA3 (TAS3) gene [122]. DCL4-mediated ta-siRNA biogenesis plays a key role in regulating rice reproductive development [122] (Fig. 19.4).

Histone lysine methylation and demethylation are important for activating and repressing gene expression. The rice JMJD2 family gene *JMJ706* encodes a heterochromatin-enriched protein that can specifically reverse the di- and trimethylations of lysine 9 on histone H3 (H3K9) in vitro. JMJ706-dependent H3K9 demethylation affects the flower development by modulating the expression of *DH1* (*Degenerated Hull1*) and *OsMADS47* [123] (Fig. 19.4). *DH1* encodes a protein with a LOB (lateral organ boundaries) domain, which is required for spikelet development (Fig. 19.4).

5 Male Reproductive Development

Male reproductive development starts from the initiation and generation of stamen within the flower. A stamen consists of an anther, which contains multiple specialized tissues for generating pollen grains, and a filament that supports the anther (Fig. 19.1j, k). Pollen development consists of a series of crucial developmental events, including male sporogenous cell differentiation, meiosis, microspore formation, and maturation, and requires cooperative interactions between gametophytic and sporophytic tissues within the anther [3, 124–130]. Recent transcriptome analyses revealed about 29,000 unique transcripts in rice anther and male gametophyte [131–138], confirming the complexity of anther development and pollen formation.

Although rice flower develops a quite different structure from that of *Arabidopsis*, its reproductive organs, i.e., stamens and carpels, share similar genetic pathway with the latter. Like *Arabidopsis*, rice stamens are also symmetrically arranged in the third whorl, and each stamen contains a filament and an anther with four lobes linked to the filament by connective tissues. Each rice anther has two thecae linked by the connective tissue, and each theca consists of two locules, the one at the base is longer. The two locules are linked by septum and stomium (consisting of small epidermal cells), which play a role during anther dehiscence [139]. Based on the well-defined cellular landmarks, rice anther development is divided into 14 stages encompassing stamen (anther) primordium formation cell division and differentiation and the formation of characteristic anther structures such as four somatic anther wall layers (the epidermis, the endothecium, the middle layer, and the tapetum) and microspore mother cells within the locule (Fig. 19.1j) [3, 130]. In microspore mother cells, meiosis occurs to form dyads and tetrads of haploid microspores, which are subsequently released from the tetrads as free microspores and become vacuolated. The vacuolated microspores undergo two rounds of mitosis, generating two sperm cells and a vegetative cell [3, 130].

Rice anthers have obvious lobe boundaries and reticulate anther cuticles. In addition, the tapeta of rice and other cereals have characteristic orbicules/Ubisch bodies, which are thought to export tapetum-produced sporopollenin precursors across the hydrophilic cell wall to the locule [140, 141]. However, orbicules have not been observed in the Brassicaceae family including *Arabidopsis*, which have unique secretory tapeta with special organelles such as elaioplasts and tapetosomes [140–143]. Rice pollen exhibits a smooth and particulate exine patterning and contains a wider inter-layer space between the nexine (foot layer) and sexine, in comparison with that of *Arabidopsis* [144, 145].

Recent forward and reverse genetic analyses greatly improved our understanding of the molecular mechanisms of male reproductive development in rice [130]. Genes such as *MSP1* (*MULTIPLE SPOROCYTE*) [146, 147], *OsTDL1A* [148], *GAMYB* [149–151], and *UNDEVELOPED TAPETUM1* (*UDT1*) [152] have been shown to control early anther development in rice.

In addition, *PAIR1* (*HOMOLOGOUS PAIRING ABERRATION IN RICE MEIOSIS1*) [153], *PAIR2* [154], *PAIR3* [155], *MEIOSIS ARRESTED AT LEPTOTENE1* (*MEL1*) [156], *OsRAD21-4* [157], *ZEP1* [158], and *PSS1* (*Pollen Semi-sterility1*) [159] have been identified as meiosis regulators. Moreover, *Tapetum Degeneration Retardation* (*TDR*) [160, 161], *PERSISTENT TAPETAL CELL 1* (*PTC1*) [162], *EAT1* [163], and *APOPTOSIS INHIBITOR5* (*API5*) are involved in postmeiotic tapetal cell death [164]. Lastly, the C-class gene, *OsMADS3*, was shown to play a key role in regulating late anther development and pollen formation via modulation of reactive oxygen species (ROS) levels [111].

During male reproductive development, the anther also synthesizes various molecules required for normal pollen formation and maturation. *Wax-deficient anther1* (*WDA1*) [165], *CYP703A3* [149], *CYP704B2* [144], *Defective Pollen Wall* (*DPW*) [166], *PDA1* [167], and *OsC6* [168] encode enzymes/proteins involved in fatty acid modification, elongation, and transportation and have been shown to be essential for the formation of lipidic anther structures, i.e., the anther cuticle and the pollen exine [130]. Carbon starved anther (CSA), a putative R2R3 MYB-type transcription factor, regulates sugar partitioning [169]. Additionally, *Rice Immature Pollen 1* (*RIP1*) and *ANTHER INDEHISCENCE1* are also required for late pollen maturation and anther development [170, 171]. Despite the importance of female organ in rice, we do not summarize the understanding of female organ formation because limited information has been revealed on this topic.

6 Conclusion and Perspective

Molecular genetic and biochemical studies have successfully identified a number of key regulators from flowering plants for the specification of reproductive organ identity. The availability of whole genome sequences, large numbers of mutants, genome-scale expression profiling data, and advanced sequencing capability will significantly speed up the identification of genes crucial for rice reproduction. The tools of cell biology and biochemistry as well as systems biology will also be essential for revealing molecular and biochemical mechanisms underlying the function of the identified genes. In addition, comparative analysis of rice reproductive regulators with those from maize, *Arabidopsis,* and other model plants will uncover the conserved and divergent regulatory pathways controlling plant reproduction. More importantly, knowledge gained from the study of these biological processes can be used in selective breeding and hybrid rice production. The future for the systematic studies of rice reproduction is very exciting.

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Root Development

20

Jian Xu and Jing Han Hong

1 Introduction

Unlike the dicotyledonous model plant Arabidopsis, which has a taproot system dominated by the primary root, rice has a fibrous root system that is developed postembryonically (Fig. 20.1). The root system architecture of rice is typical of monocotyledons in which the primary root, often termed as seminal root, is known to be ephemeral and short living. Two to three days after germination, several crown roots emerge from the coleoptilar node by breaking the sheath. Together with lateral roots, which originated from the primary root, and crown roots, these roots make up a fibrous root system. The numbers of crown roots vary between rice cultivars and increase over time, for example, in the case of the japonica cultivar Nipponbare, more than 100 crown roots are present 40 days after germination in rice plants grown under hydroponic conditions [1]. Lateral roots in rice can be classified into two main anatomical types [2]. The vast majority of lateral roots are small and thin, elongate laterally, exhibit determinate growth, and never bear lateral roots, whereas a few large lateral roots, which are thinner in diameter than seminal and crown roots and display indeterminate growth, can also be observed. These large lateral roots elongate downward in response to gravity and can also bear small lateral roots. The crown roots and lateral roots work together to anchor the rice plant to the ground, to facilitate the plant to absorb nutrients and minerals from the soil, and to allow the plant to resist biotic and abiotic stresses, so as to maximize plant survival chances and minimize loss of yield and quality.

In recent years, a number of genome-wide large-scale studies have been performed to uncover the molecular mechanisms that regulate rice root development [3, 4]. For example, Jiao et al. used laser microdissection and microarray profiling from 40 different cell types, comparing transcriptomes from different cell types to create a transcriptome atlas [3]. Previously undiscovered properties, such as cell-specific promoter motifs, interaction partner candidates, coexpressed cognate binding factor candidates, and hormone response centers, were uncovered in this method [3]. Takehisa et al. also adopted the same technique to perform an even more comprehensive analysis, by combining 38 microarray data that cover eight developmental stages along the longitudinal axis and distinct tissues along the radial axis of the crown root. They focused on root development, function of the root cap, lateral root formation, and water and nutrient absorption, in order to identify genes and gene networks associated specifically with the function and formation of the rice root system [4]. Based on

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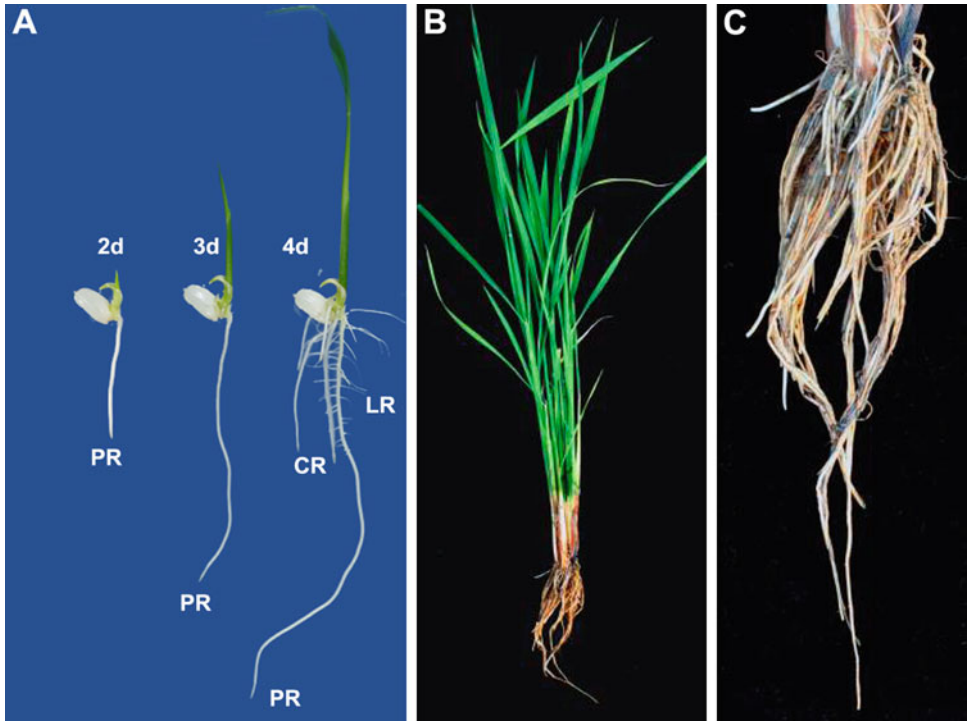

Fig. 20.1 Rice root system. (**a**) Morphology of the rice seedling, 2–4 days after germination. (**b**, **c**) Morphology of a 60-day-old rice plant (**b**) showing the fibrous root system (**b**, **c**). Many crown roots develop from the stem nodes. Lateral roots develop from both the primary and crown roots. Both small and large lateral roots are present (not shown). *PR* primary root, *CR* crown root, *LR* lateral root

expression pattern of genes involved in the biosynthesis and signaling of plant hormones, they revealed various forms of interactions between the plant hormones. Their expression profiles are easily accessible on RiceXPro, which is a comprehensive database that contains retrievable gene expression information in rice [5]. This provides researchers all over the world baseline information to discover gene regulatory networks involved in the development of rice roots and their physiological functions.

Other commonly used techniques make use of a combination of forward and reverse genetics methods, including quantitative trait loci (QTLs) analysis, ethyl methanesulfonate (EMS) mutagenesis and T-DNA insertional mutagenesis screens, gene knockdown, and overexpression experiments. This chapter summarizes the various molecular players and mechanisms that have been identified by researchers in the past 10 years (2003–2012), that play roles in rice root development, which are listed in Table 20.1.

2 Initiation of the Different Types of Rice Roots

2.1 Patterning the Radicle and Primary Root

Due to the highly variable and unpredictable cell division pattern of rice embryogenesis [6], it remains a challenge to analyze in detail the origin of the radicle (embryonic root) in the rice embryo. However, the radicle can be recognized at the ventral side of the embryo from around 5 days after anthesis (5AA), which is enclosed by a structure referred to as the coleorhiza that subtends the radicle to unsheathe and protect it during embryogenesis and upon germination.

During germination of the rice seed, the radicle elongates and becomes the primary root. Both the radicle and primary root exhibit a stereotypic cell division pattern, resulting in a highly organized

Table 20.1 A summary of the molecular players (in acronyms) involved in root development in rice

Developmental processes	Cell/tissue/root types	Molecular players	References
Root initiation	The radicle	OsCEM, CSLD1,2, CSLC3, CSLF3	[11, 18]
	Crown root	CRL1/ARL1, CRL5, OsRR1, CRL4/OsGNOM1, OsPIN2/5b/9, FAS1, GTE4, MAP, miR167	[22–26, 33, 87]
	Lateral root	CRL1/ARL1, CRL4/OsGNOM1, OsIAA3/OsIAA31, OsIAA11, OsIAA13, OsIAA23, ARM1, ARM2, miR164	[22, 23, 25, 26, 33, 38–42, 86]
Cell/tissue specification and development	Outer cell layers	C68, DOCS1, OsRHL1, OsEXPA17, OsEXPA30, RTH1, OsCSLD1, OsPHR2	[53, 58, 59, 62]
	Ground tissues	OsSHR1, OsSCR1	[70, 71]
	Root cap	GLR3.1, EUI, miR160	[75, 76, 86]
	Quiescent center	QHB, FCP1/CLE402, OsIAA23	[41, 78, 79]
Root growth	Primary root	OsARF12, OsRAA1, PHYB, PHYC, OsRMC	[80, 81, 92, 127, 128]
	Crown root	WOX11, OsRR2, OsPIN1, OsPIN3t, OsCAND1, miR393a, OsTIR1, OsAFB2, GA2oxs	[80–85, 89, 94, 128]

root structure in both longitudinal and radial directions. Although a cell lineage map for the radicle and primary root is still missing, microscopic analysis of sections of the radicle and primary root tips shows that they are made of, from the outside in, the outer cell layers, the ground tissue, and the stele. The outer cell layers comprise the epidermis, the exodermis, and the sclerenchyma. Each of them consists of a single layer of cells. The ground tissue is composed of 10–15 concentric layers of cortex cells and one endodermal cell layer [7, 8]. The stele, enclosed by the endodermis, contains a single layer of parenchyma cells called the pericycle, a polyarch central cylinder of xylem vessels and phloem bundles. In the longitudinal direction, the radicle and primary root tips of rice can be subdivided into two meristematic zones. At the distal tip of the radicle and primary root is the root cap meristem, also called calyptrogen, which consists of columella and lateral root cap stem cells and gives rise to the differentiated columella and lateral root cap cells. Located above the calyptrogen is the arc-shaped proximal root meristem that contains a small number of mitotically less-active quiescent center (QC) cells and the stem cells that divide asymmetrically to continuously produce cells of the outer cell layers, the ground tissue, and the stele (Fig. 20.2).

The initiation of the radicle may be modified by changes in responses to hormones or hormonal levels or by genes that define the axis and initiation of the organs. For example, the recessive *radicleless1* mutant [9], which lacks the radicle and thus the primary root but can still produce crown roots and lateral roots after germination, was shown to have a defective response to auxin and an enhanced sensitivity to cytokinin [10]. In addition, a newly discovered mutant *OsCem* was observed to develop Siamese polyembryos with multiple radicles [11]. Roughly mapped to chromosome 3, between molecular markers M148 and RM468, the *OsCem* gene is believed to encode an embryo-specific protein involved in polar auxin transport [11]. Auxin level in the *OsCem* mutant embryo was detected to be higher as compared to wild-type embryos [11]. More work is needed to understand the detailed molecular mechanism of how *RADICLELESS1* and *OsCem* affect initiation of the radicle.

Members of the cellulose synthase and cellulose synthase-like gene superfamily (CESA/CSL) are known to encode enzymes to synthesize

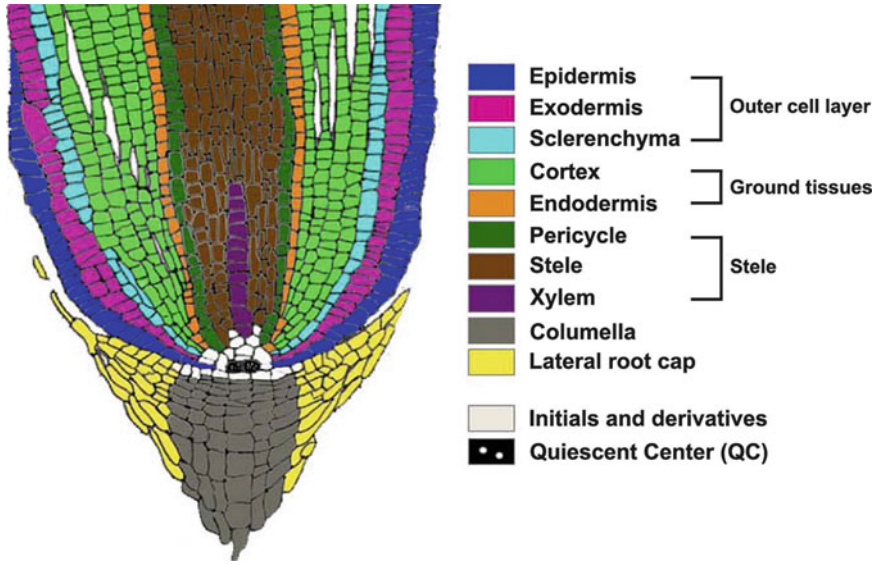

Fig. 20.2 Organization of the primary root meristem, median longitudinal view. Cell types are color coded as indicated (from Coudert et al. [33], reprinted with permission)

cellulose and non-cellulosic matrix of the cell wall in plants [12–17]. Forty-five OsCESA/CSL members were identified in the rice genome [18]. Using microarray analysis performed with 33 tissue samples of two rice varieties, followed by phylogenetic analysis, structural analysis, and co-expression profiling, it was uncovered that some of the members of the CSL family such as CSLD1,2, CSLC3, and CSLF3 are preferentially expressed in the radicle and subsequently primary root [18]. These specific OsCESA/CSLs may therefore play important roles in the initiation and development of the radicle.

2.2 Crown Root Initiation

A large part of the rice root system is made of crown roots, which are post-embryonic roots mainly initiated from the lower stem nodes [19]. The crown roots provide anchorage for rice plants to tolerate and survive overextended periods of flooding and play an important and major role in allowing the plant to absorb water and nutrients from the soil. The process of crown root formation in rice has been known for a long time since the classic histological work of Kaufman [20] and Kawata and Harada [21]. Itoh et al. [6] divided the entire process into seven successive stages (Fig. 20.3) with a modification to that by Kawata and Harada. Rice crown roots originate from crown root founder cells, which are formed in the pericycle (Stage 1). These founder cells then undergo two rounds of periclinal divisions to generate a three-layer primordium comprising initial cells for the stele, epidermis–ground tissue, and root cap, respectively (Stage 2). The initial cells for epidermis–ground tissue subsequently divide periclinally to form one epidermal layer and one ground tissue layer (Stage 3), whereas the initial cells for the stele and root cap expand radially through anticlinal and periclinal divisions, leading to the dome shape of the crown root primordium (Stages 3 and 4). The cells in the ground tissue layer further divide periclinally to produce several layers of cortex cells and one endodermal cell layer (Stage 4). The fundamental organization of the crown root becomes established at Stage 5, at which the columella root cap is formed and, in the central region of the stele, a large metaxylem vessel starts to appear. During Stage 6, cells in the basal region of the stele show commencement of cell elongation and vacuolation, and those of the

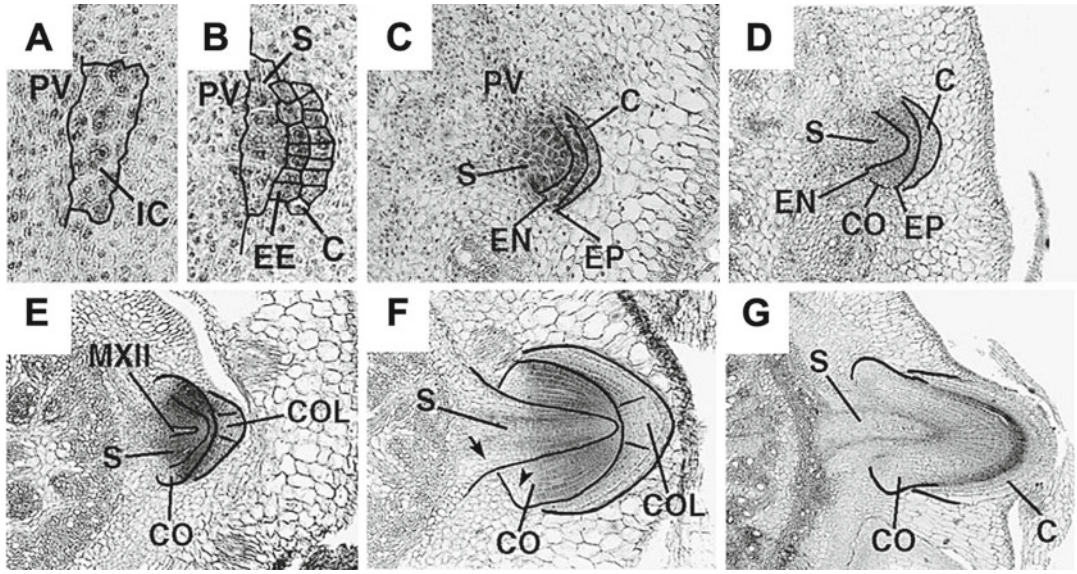

Fig. 20.3 The development of the crown root. (**a**) Stage 1: The initial cells are established. (**b**) Stage 2: The root cap initials and epidermis–endodermis initials are established. (**c**) Stage 3: The epidermis and endodermis differentiated from the epidermis–endodermis initials. (**d**) Stage 4: The cortex differentiates. (**e**) Stage 5: The organization of the root primordium is established. (**f**) Stage 6: The cells in the cortex undergo vacuolation (*arrows*) and the stele elongates. (**g**) Stage 7: The crown root emerges. *C* root cap or its initials, *CO* cortex, *COL* columella, *EE* epidermis–endodermis initials, *EP* epidermis, *EN* endodermis, *IC* initial cells, *PV* peripheral cylinder of vascular bundle, *S* stele (from Itoh et al. [6], reprinted with permission)

cortex show vacuolation. The root apex reaches the stem epidermis, and eventually, in the basal region of the root primordium, cells of all tissues elongate and the crown root emerges from the parental stem (Stage 7).

To date only a few rice genes involved in the crown root formation have been identified (Table 20.1). Among them, *CROWNLESS ROOT 1* (*CRL1*) [22]/*ADVENTITIOUS ROOTLESS 1* (*ARL1*) [23] is an auxin- and ethylene-responsive gene with expression in parenchyma cells adjacent to the peripheral vascular cylinder of the stem, where crown roots initiate, and encodes a plant-specific LATERAL ORGAN BOUNDARIES DOMAIN (LBD)/ ASYMMETRIC LEAVES2-LIKE (ASL) transcription factor required to activate the initiation of crown roots. *CROWNLESS ROOT 5* (*CRL5*) encodes a member of the APETALA2 (AP2)/ ETHYLENE RESPONSIVE FACTOR (ERF) transcription factor family and was also shown to be auxin-induced and expressed in the stem nodes region where crown root initiation occurs [24]. Like *crl1* mutant, *crl5* mutant produces much fewer crown roots than wild type, and an additive phenotype was seen in a *crl1 crl5* double mutant [24], indicating that *CRL5* functions in a different pathway to *CRL1*/*ARL1*. Consistently, *CRL5* was found to repress cytokinin signaling at least through its promotive effect on the transcription of *OsRR1*, encoding a type-A response regulator of cytokinin signaling [24].

Additional molecular players that regulate auxin signaling, transport, and distribution have also been shown to influence the initiation of the crown root. For example, rice plants with mutations in *CROWNLESS ROOT 4* (*CRL4*) [25]/*OsGNOM1* [26] are defective in the formation of crown root primordia. *CRL4*/*OsGNOM1* is expressed in the crown root primordia [25, 26] and encodes an ADP-RIBOSYLATION FACTOR-GUANIDINE EXCHANGE FACTOR (ARF-GEF) homologous to Arabidopsis GNOM, which is known to modulate polar auxin transport through its effect on the intracellular trafficking of PINFORMED1 (PIN1) auxin efflux carrier protein [27–29]. Similarly in *Osgnom1* mutants, *PINFORMED2* (*OsPIN2*), *PINFORMED5b*

(*OsPIN5b*), and *PINFORMED9* (*OsPIN9*) expression is altered as shown by quantitative RT-PCR approaches [26]. Thus, it is likely that OsGNOM1 mediates *OsPINs* expression to modulate crown root initiation [26].

Furthermore, using methods of transcript profiling in *crl1* mutants, out of 486 genes identified, three early auxin-responsive genes, *FLATENNED SHOOT MERISTEM* (*FSM*)/*FASCIATA1* (*FAS1*) [30, 31], *GENERAL TRANSCRIPTIONAL FACTOR GROUP E4* (*GTE4*) [32], and *MICROTUBULE-ASSOCIATED PROTEIN* (*MAP*), have been isolated as putative targets of CRL1 that are involved in crown root initiation [33]. The knockout mutants of these genes have reduced number of crown roots [33], indicating that crown root initiation involves chromatin remodeling (FSM/FAS1 and GTE4) and the control of cell division (MAP).

In addition, Xiong et al. reported that a critical concentration of endogenous nitric oxide is indispensable for crown root initiation in rice [34]. They proposed that auxin induces an accumulation of nitric oxide, which in turn triggers a cGMP-dependent or cGMP-independent MAPK cascade, thus leading to crown root initiation and emergence [35].

2.3 Lateral Root Initiation

Similar to crown roots, both large and small lateral roots in rice originate from the pericycle. However, the endodermal cells of the parental root appear to have a yet unclear role during lateral root formation. While the epidermis, the ground tissue, the columella root cap, and the stele arise from the pericycle, the endodermal cells of the parental root may be involved in the formation of lateral root cap [2, 36, 37] (Fig. 20.4). The development of large and small lateral root primordia is similar to each other except that, at a very early stage, cortex cell layers are produced via periclinal divisions in the large lateral roots, but not in the small lateral roots [2, 37]. The physiological function of these two types of lateral roots remains unclear.

A number of genes involved in the initiation of crown root have also been associated with lateral root formation. Expression of *CRL1*/*ARL1* and *CRL4*/*OsGNOM1* was detected in tissues where lateral roots are initiated and mutation in these two genes resulted in a reduced number of lateral roots in rice plants. Studies on the *AUX*/*IAA* genes *OsIAA3* (now named as *OsIAA31*) [38], *OsIAA11* [39], *OsIAA13* [40], and *OsIAA23* [41] further suggest that auxin signaling can influence lateral root initiation. When a stabilizing mutation of these auxin repressors is overexpressed, lateral root initiation is inhibited [38–41]. Furthermore, through screening for auxin-resistant mutants, two putative mutants have been identified, named *arm1* and *arm2*. The double mutant produced fewer lateral roots [42].

In addition to auxin, other molecules such as nitric oxide, methyl-jasmonate, and hydrogen peroxide were also shown to promote lateral root initiation in rice through calcium signaling [43–45].

3 Specification and Development of the Different Root Tissues

3.1 Outer Cell Layers of Rice Roots

The outer cell layers of the rice root are made up of the epidermis, exodermis, and sclerenchyma [46–48] (Fig. 20.2), which is a constitutive feature present in hydroponically and aeroponically grown roots and in both upland (dryland) and paddy rice varieties [49]. The epidermal layer is the outermost layer that is in direct contact with the soil environment. As the root develops, root hairs emerge from the epidermis, and dead cells at the maturation zone of the epidermis are sloughed off, exposing the underneath cells of the exodermal layer which will function like the epidermal cells with regard to water and nutrient uptake. A single layer of sclerenchyma fiber tissue is laid down below the exodermis [50]. The cytoplasm of sclerenchyma cells is rarely observed, suggesting that they are dead [47, 51].

The epidermis of rice roots comprises hair (H) cells and non-hair (N) cells. H cells are shorter

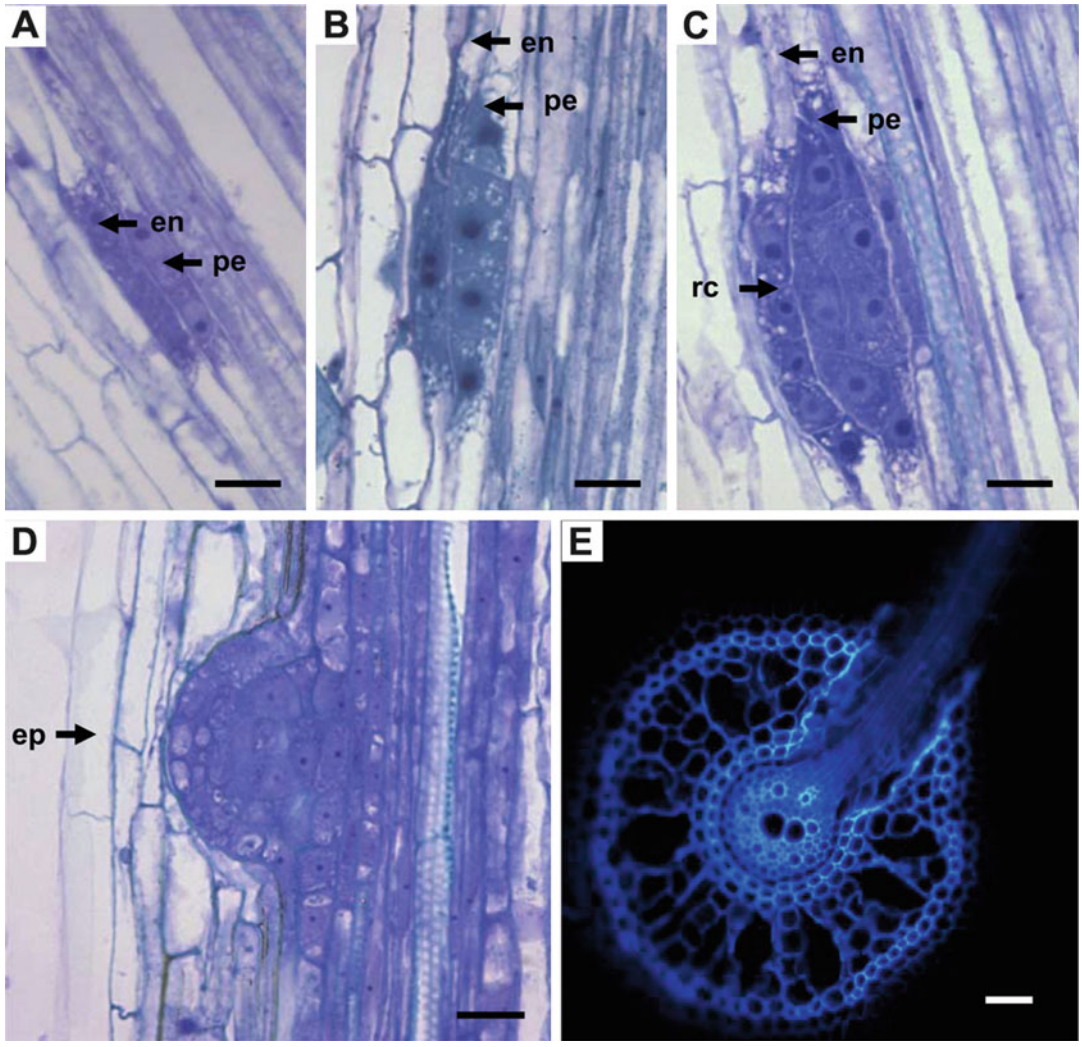

Fig. 20.4 Stages of small lateral root primordium development. (**a**) First anticlinal cell division in the pericycle. (**b**) Two additional anticlinal divisions in the inner cell layer of the small lateral root primordium. (**c**) Periclinal divisions have divided the inner layer into two layers and lateral root cap has formed in the endodermis. (**d**) Emerging small lateral root primordium. (**e**) Fully emerged small lateral root. *en* endodermis, *pe* pericycle, *rc* future lateral root cap, *ep* epidermis. Scale bars (**a**–**e**) 25 μm (from Rebouillat [36], reprinted with permission)

than N cells at maturity [52, 53] but have the same size as N cells at hair initiation, indicating that H and N cells are derived from a symmetric cell division in rice and the difference in H and N cell size observed at maturity arises from differential cell expansion in H and N cells after root hair initiation [54] (Fig. 20.5). H cells in rice are thought to be important in water and nutrient uptake.

Cells at the maturation zone (approximately 50 mm from the root apex) of the exodermal layer possess Casparian bands and have fully mature suberin lamellae, which are weakly developed in the exodermis at a distance of around 20 mm from the root apex. Suberin is however absent in cells of the heavily lignified sclerenchyma layer. At a given distance from the root apex, more lignin can be observed in the exodermis layer than in the sclerenchyma layer [47]. Both Casparian bands and suberin are known as apoplastic barriers that do not function

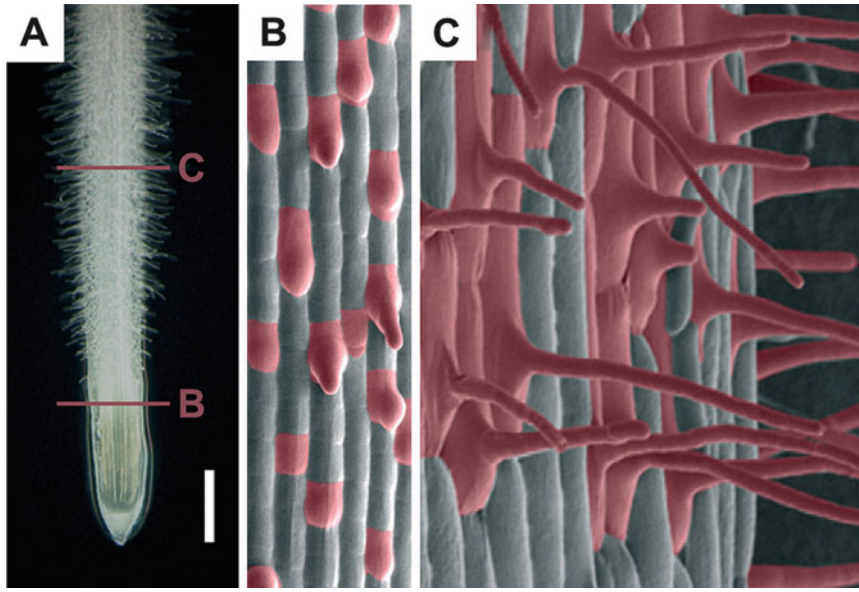

Fig. 20.5 Root epidermal development. (**a**) A 3-day-old seminal root imaged with dark field illumination. *Red color lines* correspond to the position of images in (**b**) and (**c**). (**b**, **c**) Cryo-scanning electron microscope image of (B) the zone of root hair initiation and (C) the zone where root hairs are almost fully grown. *Red color* highlights H cells. Scale bars (**a**) 500 μm (from Kim and Dolan [54], reprinted with permission)

in an all-or-none fashion. Rather, they exhibit a selectivity pattern and provide an adaptive mechanism in response to conditions in a given habitat such as drought, anoxia, salinity, heavy metal, or nutrient stresses [55]. In rice roots, there is substantial apoplastic transport of water across the outer cell layers, even in the presence of suberized exodermis and a thick layer of sclerenchyma cells. This is differing from that found for oxygen [56, 57], whose permeability is depending on suberization of the exodermis and lignification of sclerenchyma, suggesting that the two diffusants use different pathways with the outer cell layers.

Although the outer cell layers of rice roots play important roles in protecting the roots from various forms of stresses in soil, the molecular mechanisms for the specification and development of these cell layers are poorly understood. *c68* is a mutant that is sensitive to aluminum, lanthanum, and cadmium, as there is a greater penetration of these metals into the inner cell layers [48]. This mutant was observed to have, at a region close to the root meristem, disordered pattern of additional periclinal cell divisions. Although the inner cell layers and root meristem of this mutant appeared to be normal, this mutant has less root hairs, smaller and irregular epidermal cells, and additional sclerenchyma-like cells transformed from exodermal cells [48]. The total number of lateral roots is normal in this mutant, but there are more large lateral roots; the small lateral roots displayed abnormalities such as curved or wavy shape and are shorter in length as compared to wild-type rice [58]. Using this mutant and a map-based cloning approach, *DEFECTIVE IN OUTER LAYER SPECIFICATION 1* (*DOCS1*) gene was isolated and identified to be one of the factors responsible for the proper specification and development of rice outer cell layers [58]. *DOCS1* encodes a leucine-rich repeat receptor-like kinase and was found to be polarized locally at the plasma membrane at the distal side of the cells in the outer cell layers [58]. Microarray analysis showed that genes encoding putative transcription factors and genes that may be involved in cell wall metabolism are altered in this mutant, suggesting that *DOCS1* may regulate a cascade of genes that is needed to properly specify and develop the outer cell layers of the rice root [58].

Two allelic mutants defective in root hair elongation were isolated in an EMS mutagenesis screen and designated as *Osrhl1-1* and *Osrhl1-2* [53]. *OsRHL1* gene has been identified that encodes a member of the subfamily C of the rice basic-helix-loop-helix transcription factor, and its expression is root hair-specific [53]. Cryo-scanning electron microscopy (cryo-SEM) images suggest that *OsRHL1* can also control epidermal cell patterning in the rice root [53]. Other mutants with shorter root hairs that were isolated showed mutations in various root hair-specific cell wall-loosening expansin genes such as *EXPANSIN A17* (*OsEXPA17*) and *EXPANSIN A30* (*OsEXPA30*) [59] and in the *ROOT HAIRLESS1* (*RTH1*) gene [60] encoding an enzyme apyrase that can hydrolyze NTPs and/or diphosphates. A *CELLULOSE SYNTHASE-LIKE D1* (*OsCSLD1*) gene has been also shown to be involved in root hair development but not initiation; *Oscsld1* mutants display abnormal root hair with kinks and swellings [61]. In addition, *PHOSPHATE STARVATION RESPONSE 2* (*OsPHR2*), encoding a member of the MYB-CC family of transcription factors, was shown to be involved in a phosphate-mediated regulatory pathway in root hair growth [62].

3.2 Ground Tissues

Depending on the root type, ground tissue in rice is formed by a single endodermal layer (small lateral roots) or by multiple layers of cortex and the endodermis (the radicle, primary root, crown roots, and large lateral roots). Generally, large lateral roots are thinner, with a lower number of cortex layers, whereas the radicle, primary root, and crown roots are considerably thicker, with multiple layers of cortex.

At a position proximal to the root meristem, cortex cells start to collapse and will gradually differentiate into a lysigenous–schizogenous aerenchyma with intercellular spaces that develop into large lacunae [36] (Fig. 20.6). As a result, fully developed aerenchyma separates the inner part of the root from the outer cell layers at the maturation zone. Cell collapse occurs in a specific location in the root cortex [63], where cells expand greatly and lose contact tangentially with neighboring cells, generating the so-called schizogenous spaces between cells. Later, these cells and their neighboring cells in the radial direction collapse and produce cellular spaces called lysigenous spaces. Not all the cortex cells collapse; the outermost and innermost cells remain intact.

Aerenchyma can be formed constitutively or induced by abiotic stresses, usually by hypoxia that results from waterlogging. Ethylene has also been shown to increase root aerenchyma formation [64, 65]. Aerenchyma is defined as a tissue that is devoted to gas exchange between the shoot and the root and acts as a reservoir of oxygen that is required for root respiration in paddy fields. Aerenchyma is also important as it is involved in the release of a greenhouse gas, methane, to the atmosphere in flooded soils. Therefore, aerenchyma formation has been intensively investigated in rice and other plant species [66].

The endodermis of rice root is the innermost layer of the ground tissue. As typical for most roots, endodermal cells in rice roots exhibit cell wall modifications, corresponding to three different developmental states of the endodermis [67]: State 1 endodermis starts to develop Casparian bands in the radial cell walls at distances closest to the root tip; State 2 endodermis has also suberin lamellae laid down on the inner walls of endodermal cells; and State 3 endodermis is characterized by the deposition of lignified cellulose walls on the suberin lamella at distances closest to the root base. Endodermal cells represent a resistant apoplastic barrier to water and nutrition flow.

The formation and development of ground tissue layers in Arabidopsis roots requires the function of two related GRAS family transcription factors SHOOTROOT (SHR) and SCARECROW (SCR) [68, 69]. Like in Arabidopsis, *SHORTROOT 1* (*OsSHR1*) is also expressed in the stele and *SCARECROW 1* (*OsSCR1*) is in the endodermis [70, 71], which suggests a similar mechanism controlling the formation of ground tissue layers, though mechanisms to control additional divisions in the ground tissue are still unknown [72].

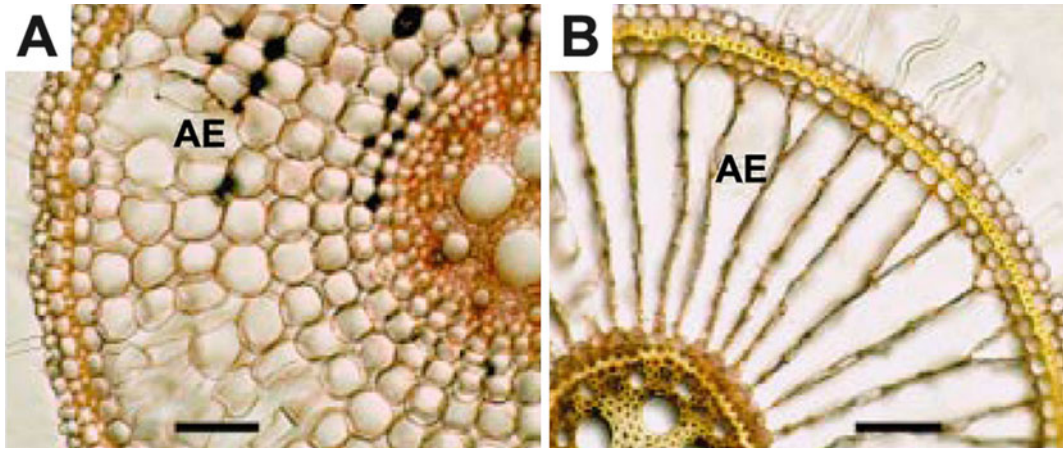

Fig. 20.6 Aerenchyma formation. (**a**, **b**) Development of aerenchyma at 20 (**a**) and 100 mm (**b**) from the apex of roots of 30-day-old rice plants. *AE* aerenchyma. Scale bars (**a**, **b**) 100 μm (from Ranathunge et al. [47], reprinted with permission)

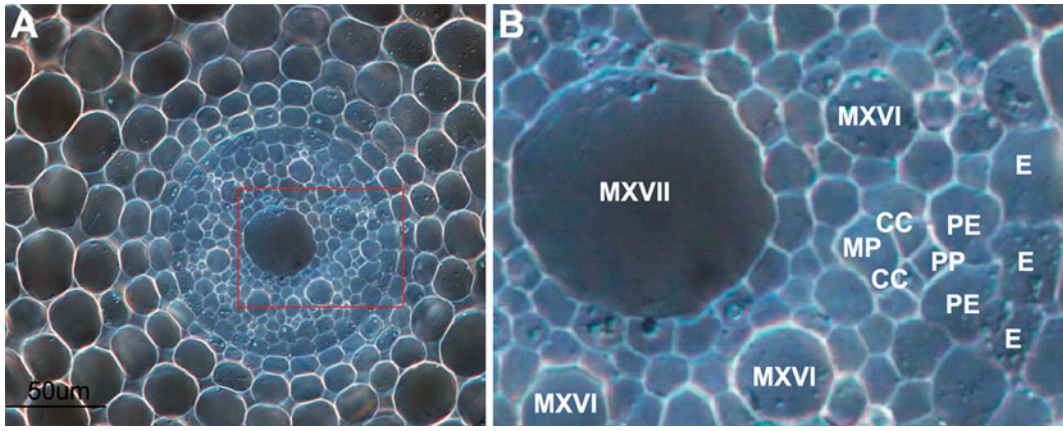

Fig. 20.7 The stele of the primary root. (**a**) Cross section of the primary root. (**b**) Boxed region in (**a**). *CC* companion cell, *E* endodermis, *MP* metaphloem sieve tube, *MXVI* early metaxylem vessel, *MXVII* late metaxylem vessel, *PE* pericycle, *PP* protophloem sieve tube

3.3 Stele

The stele of rice roots consists of a polyarch central cylinder of xylem vessels and phloem bundles surrounded by a layer of pericycle cells (Fig. 20.7). In transverse section, the xylem of rice roots typically has small distinctive ridges of protoxylem along the margin. The differentiation of xylem and phloem proceeds centripetally. A subset of pericycle cells are involved in the formation of lateral roots in rice. The large lateral root has only one late metaxylem vessel in the center of the stele, but this is missing in a small lateral root. The structure of lateral roots lacking later metaxylem vessel is poorly developed [8].

The stele is very important for the absorption and translocation of water and nutrients. In particular, the size and number of xylem vessels in rice roots significantly affect water transport from root to shoot [73]. In both hydroponic culture and under field conditions, upland cultivars were shown to have larger stele and late metaxylem vessel diameters than lowland rice cultivars [73]. The genetic factors controlling natural variation of stele and xylem structures in rice remain unknown. However, at least eight QTLs have

been detected in an F3 population from a cross between an upland cultivar and a lowland cultivar [74]. The map information for these QTLs shall pave the way for improvement of stele and xylem structures through marker-assisted selection, eventually leading to the identification of underlying mechanisms of natural variation, though how to evaluate the stele and xylem structures effectively remains a challenge.

3.4 Root Cap

The root cap in rice is divided between the apical part (columella) and the lateral part (lateral root cap). Unlike in Arabidopsis, lateral root cap in rice does not originate from the epidermis/lateral root cap stem cells, instead, lateral root cap and columella root cap in rice may originate from the same initials. If the complete root cap is removed, the root is able to regenerate a new root cap. The columella root cap contains statocytes that are involved in gravity perception. In addition, root caps in rice could perceive and process many other environmental stimuli including light, temperature gradients, humidity, obstacles, and ions.

In a T-DNA insertional mutant of a rice glutamate receptor-like gene *GLR3.1* [75], the number of lateral root cap cell layers is significantly increased compared to the wild type. Bromodeoxyuridine (BrdU) incorporation assay indicates that the mitotic activity of lateral root cap stem cells is greatly enhanced in the mutant, which could account for the lateral root cap phenotype. By contrast, a similar division pattern of columella stem cells was found in the wild type and *glr3.1* mutant, suggesting that rice glutamate receptor-like gene *GLR3.1* has a disparate effect on cells of differing origins in root cap development.

In addition, gibberellins may play roles in starch granule development and gravity responses in the root cap. Mutations in *ELONGATED UPPERMOST INTERNODE* (*EUI*), encoding a cytochrome P450 monooxygenase involved in the deactivation of gibberellins, were shown to affect starch granule development in the root cap [76].

3.5 QC

The QC plays an important role in root meristem maintenance by regulating the differentiation of adjacent stem cells and serving as a source for replenishing expiring stem cells [77]. *QUIESCENT-CENTER-SPECIFIC HOMEOBOX* (*QHB*), an ortholog of the QC-specific gene *WUSCHEL-RELATED HOMOBOX 5* (*WOX5*) in Arabidopsis, is specifically expressed in the QC of the rice root meristem [78], suggesting an evolutionally conserved role for *WUSCHEL*-type homeobox genes. When a synthetic peptide of FLOWER ORGAN NUMBER2 (FON2)-LIKE CLE PROTEIN (FCP1/CLE402) is exogenously applied to rice roots, the root meristems are consumed with the cell fates in the root meristem misspecified; the expression domains of *QHB* and *OsSCR1* expand proximal to the root meristem and Histone H4 expression is detected in the QC region which should express QHB [79]. Therefore, FCP1/CLE402 likely regulates QC specification and stem cell maintenance in the rice root meristem [79].

Aux/IAA-based auxin signaling also plays roles in the maintenance of the QC. In a semi-dominant mutant of *OsIAA23*, auxin signaling is absent in the QC region, and consequently, QC identity is lost in this mutant [41]. *OsIAA23* is specifically expressed in the QC of primary, lateral, and crown roots [41], suggesting a cell-autonomous role for *OsIAA23* in the QC specification during post-embryonic development [41].

4 Hormonal Control of Root Growth

4.1 Auxin

Auxin is able to regulate the growth and thus the length of the primary root of rice. When *AUXIN RESPONSE FACTOR 12* (*OsARF12*), encoding a transcription factor required for the activation of auxin-inducible genes, is knockout in T-DNA insertional mutants, primary root elongation is decreased [80]. In *Osarf12* knockout plants, the

expression of most of the *OsPINs* and *PHOSPHOGLYCOPROTEINS* (*OsPGPs*) is found to be significantly reduced [80], suggesting that *OsARF12* acts through regulating the expression of auxin transport proteins to control root elongation.

Auxin may also control the growth of the primary root by regulating cell division. Overexpression of an auxin-inducible gene *ROOT ARCHITECTURE ASSOCIATED1* (*OsRAA1*) could strongly affect the mitotic cycle and reduce the primary root growth in rice [81]. Interestingly, overexpression of *OsRAA1* also caused endogenous indole-3-acetic acid to increase [81], suggesting that a positive feedback loop between *OsRAA1* and auxin controls the primary root growth.

Auxin signaling and transport can also stimulate the emergence and growth of the crown root. For example, PINFORMED1 (OsPIN1), a putative auxin efflux protein, may play an important role in auxin-dependent crown root emergence [82]. PINFORMED3t (OsPIN3t), another putative auxin efflux protein, has been suggested to play roles in the development of the crown root to confer tolerance to rice in event of drought stress, though OsPIN3t may work together with other OsPINs to exert its effect [83]. Nevertheless, when OsPIN3t is overexpressed, the number and length of the crown root increases [83]. In addition, CULLIN-ASSOCIATED AND NEDDYLATION-DISSOCIATED1 (CAND1), which regulates SCF^{TIR} (SKP1P, CDC53P/CULLIN, and F-BOX protein complex) assembly and disassembly, known as OsCAND1 in rice, was also shown to regulate auxin signaling and influence the emergence of the crown root through maintaining G2/M cell cycle transition [84]. Likewise, miR393a, expressed mainly in the crown and lateral root primordia, affecting auxin signaling by modulating expression of rice *TRANSPORT INHIBITOR RESPONSE 1* (*OsTIR1*) and *AUXIN SIGNALING F-BOX 2* (*OsAFB2*), also affects the growth of the primary root and crown roots [85]. Auxin signaling can also be influenced by several families of microRNAs, which target the *AUXIN RESPONSE FACTORS* (*ARFs*). For example, overexpression of *miR160* (of which *ARF* targets are still unknown) results in root cap defects in rice [86]. In the auxin-insensitive mutant *auxin resistance* (*Osaxr*), the expression of *miR164* family was observed to be significantly increased [86] and that of *miR167* significantly repressed [87]. The *Osaxr* mutant was observed to have a wide range of root defects including decreased formation of the lateral roots and crown roots [87].

4.2 Cytokinin

Cytokinin is a negative regulator of crown root development. When the cis form of cytokinin was overproduced by overexpressing putative *cis*-zeatin-*O*-glucosyltransferase in rice, it was observed that the development of crown roots was inhibited and the number of crown root decreased [88].

Moreover, *WUSCHEL-RELATED HOMEOBOX 11* (*WOX11*), expressed in emerging crown roots and later in cell division regions of the root meristem, was shown to directly repress *OsRR2*, a type-A cytokinin-responsive regulator gene expressed in crown root primordia [89]. In *wox11* mutants and *WOX11* RNA interference transgenic plants, the development of the crown roots is inhibited or delayed; whereas overexpression of *WOX11* induced precocious crown root growth and dramatically increased the root biomass by producing crown roots at the upper stem nodes and the base of florets [89]. These data suggest that *WOX11* plays a key role during crown root development. Intriguingly, *wox11* mutants are insensitive to auxin treatments to stimulate crown root development [89]. The expression of *WOX11* could be induced by both auxin and cytokinin, and the transcriptions of auxin- and cytokinin-responsive genes are affected in *WOX11* overexpression and RNA interference transgenic plants [89], indicating that, other than affecting cytokinin signaling by repressing *OsRR2*, *WOX11* could be part of the positive feedback loop of auxin signaling.

Notably, it was shown that the *CRL5* does not regulate *WOX11* expression, and these two genes are expressed at different stages of crown root

initiation, with *CRL5* at the early stages of initiation and WOX11 largely at emerging primordia and later growth [24].

4.3 Ethylene

Ethylene can affect the growth of crown roots. After treatment with ethylene, induction of the growth of crown roots is observed, with increased expression of various cell cycle markers at crown root primordia, even at early developmental stage [90]. When the crown root emerges from the nodes, the epidermal cells of the nodes die, so as not to damage the growing root [91]. In deepwater rice, it has been shown that the death of the epidermal cells can be induced by submergence as well as in the presence of the natural precursor of ethylene (1-aminocyclopropane-1-carboxylic acid), but not in the presence of ethylene action inhibitor 2,5-norbormadiene [91]. Thus, in response to low oxygen stress, both epidermal cell death and crown root growth are linked to ethylene signaling in root [91].

4.4 Jasmonic Acid

Jasmonic acid can also affect rice root development. ROOT MEANDER CURLING (OsRMC) is a jasmonic acid-induced putative receptor-like protein of the DUF26 subfamily [92]. Like its name suggests, when this gene is knocked down by RNA interference approach, the primary roots of rice curled more easily and meandered; the primary roots of the RNA interference transgenic line are shorter, the number of crown roots increased, and lateral roots decreased [92].

4.5 Abscisic Acid

When abscisic acid is exogenously added, it is observed that the crown roots swell and have more root hairs and enhanced initiation of lateral root primordia, and the cortex cells increase in size and become irregular shaped [93]; abscisic acid increases cytoplasmic Ca^{2+} levels from both extracellular and intracellular sources, which will in turn increase the number of active Ca^{2+}-calmodulin complexes and activate the Ca^{2+}-calmodulin signaling pathway. Eventually, the phosphorylation status of the ACTIN DEPOLYMERIZATION FACTOR will increase, resulting in cytoskeleton rearrangement, especially the actin filaments, which may function to regulate root hair formation, root tip swelling, and lateral root formation [93].

4.6 Gibberellins

Gibberellins are also involved in crown root growth. When gibberellins 2-oxidases (GA2oxs) that inactivate endogenous gibberellins are overexpressed, early and increased crown root growth is observed [94].

5 Environmental Regulation of Root Growth

5.1 Silicon

Silicon is a beneficial element that can improve growth yield, mechanical strength, and especially improve resistance to biotic and abiotic stress, salinity, metal toxicity, and drought stress [95–97]. In rice that is grown without silicon, the root exodermal cell walls were observed to have no suberin at early stage of development; at later stages, only about 80 % of half of the anticlinal cell wall is suberized [98]. Suberin and lignin are needed in the exodermis and sclerenchyma in order to form a barrier against diffusion of oxygen from the root to the anaerobic rhizosphere, known as radial oxygen loss, which is a critical marker for adaptation of roots to anaerobic conditions [99].

It is still not understood how rice can uptake high levels of silicon by the roots. When a gene from the aquaporin gene family 5 known as *LOW SILICON RICE 1* (*LSI1*), which is expressed at the Casparian strips (distal side of exodermis and endodermis cells), is suppressed, reduced silicon uptake was observed [97, 100, 101]. This shows

that aquaporin proteins are likely to be important for silicon uptake. In addition, using two mutants, *rh2* and *rm109*, that are defective in formation of root hairs and lateral roots respectively, it was shown that lateral roots are involved in silicon uptake in the rice plant but not root hairs [102].

5.2 Phosphorus

Phosphorus is a component of nucleic acids and energy pathways and is thus essential for normal cell division and growth. Various adaptation mechanisms to uptake and transport phosphorous under phosphorus-deficient conditions have already been reviewed [103]. Rice roots can only uptake soluble form of phosphorus.

Changes in root architecture, such as root elongation, are among the different responses of rice to phosphorus-deficient conditions; the elongated root will have higher porosity and thus oxygen release ability under these conditions [104]. Through QTL analysis, phosphorus-deficiency tolerance-specific protein kinase gene, named *PHOSPHORUS-STARVATION TOLERANCE 1* (*PSTOL1*), has been identified. *PSTOL1* is an enhancer of early root growth and its overexpression results in improvement in grain yield when the transgenic rice is grown on phosphorus-deficient soil [105].

Phosphorous-induced signaling is still largely unknown in rice. However, it has been reported that a *PHOSPHATESTARVATIONRESPONSE1*(*PHR1*)-*miR399-PHOSPHATE OVERACCUMULATOR 2* (*PHO2*) pathway, similar to that in Arabidopsis, may be involved in rice adaptation to low-phosphorous growth conditions [106].

5.3 Nitrogen

Nitrogen is one of the major nutrients that are required for the growth of rice. Nitrogen is required for biological processes such as protein synthesis, carbon and amino acids metabolism and is a component of key molecules such as nucleic acids, amino acids, and hormones [107, 108]. Genome-wide studies are useful to elucidate how rice roots response to and cope with different nitrogen levels. Cai et al. analyzed the dynamics of the rice transcriptome under nitrogen starvation conditions using Affymetrix GeneChip rice genome arrays and found 10.88 % of the transcriptome changed [109]. The genes modulated by nitrogen starvation are largely from cellular metabolic pathways such as development, molecular transport, stress response, and primary and secondary metabolism [109]. Analysis of microRNA transcriptional profiles found altered expression of *miR399* and *miR530*. Furthermore, *miR164* was observed to be significantly repressed in nitrogen-starved rice [86]. These signify the importance of miRNAs in regulating rice root system architecture and responses to the nitrogen levels in the growth environment.

5.4 Potassium

Potassium is important in many physiological processes in cells, such as osmoregulation and control of turgor pressure [110]. Rice grown under potassium-deficient conditions displays decreased shoot and root biomass [111]. Using a microarray approach, 33 hormone-related genes were shown to have altered expression in potassium-deficient rice roots. Majority of these genes are auxin-related, showing that auxin plays important roles in adaptation to potassium-deficient conditions [111].

5.5 Salinity

Rice is sensitive to salinity and different varieties have different tolerance to salinity. A more salinity-tolerant variety, such as Pokkali, has more hydrophobic barriers in its roots as compared to a more sensitive one such as IR20. These barriers are found in the root exodermis and endodermis to prevent external fluid from directly entering the stele; however, Na^+ can bypass these barriers. This can be overcome by depositing more suberin to strengthen apoplastic barriers (Casparian bands) in the endodermis and exodermis [112]. Furthermore, it has been shown that sodium

uptake is reduced in rice seedlings under salinity stress when silicon is deposited in the exodermis and endodermis, as there will be a reduction in apoplastic transport across the root [113].

RICE SALT SENSITIVE1 (RSS1) allows root meristematic cells to survive under salinity conditions. The stability of the RSS1 is controlled by cell cycle phases, and RSS1 exerts its effects by interacting with a protein phosphatase mediated by cytokinin to regulate G1 to S phase transition to ensure cell division activity under salinity stress conditions [114].

5.6 Water Availability

One of the most damaging stress to rice is drought stress, as rice, which is normally grown under flooded conditions, has a root distribution that is shallow and a limited ability to extract water from deep soil layers [115, 116]. Upland rice varieties have a deeper rooting than lowland ones and can increase water uptake from deeper soil layers under conditions of drought stress [117]. A combination of a near-vertical growth axis and increased root length along that axis is essential for the development of deep root [118].

Currently, using a "basket" method to quantify root growth angle in different varieties of rice with different resistance to drought and QTL analysis, the identification of genes that determine deep rooting in rice, in terms of root growth angle, is in progress [119–121]. A summary of the QTLs detected can be found in Courtois et al. [122].

Drought stress can also arise due to increase evaporative demand. However, it is shown that the main origin of drought sensitivity in many rice varieties is due to their poor root system [123].

It was observed that the development of lateral roots and transport of labeled auxin from the shoot to the root, in seedlings grown under saturated humidity (SH), are greatly enhanced as compared to seedlings grown under normal humidity (NH) [124]. Decapitation reduces the lateral root density, which can be restored by the addition of auxin (IAA) to the cut stem [124]. Since naphthylphthalamic acid (NPA)-induced reduction of polar auxin transport in the shoot did not reduce the lateral root density in SH seedlings, it is likely that the phloem is involved in the leaf to root auxin transport and high humidity increases the rate of transport, resulting in the increase of lateral root density [124].

In rice, *miR169g*, which is a member of the *miR169* family, has been found to be significantly induced in the roots under conditions of drought stress [125]. The mechanism how *miR169g* can remodel the root system during drought stress is still unknown.

5.7 Light

When rice root is exposed to light, it is observed that all the crown and lateral roots bend away from light and the curvature is higher for the crown root of a higher node than that from a lower node [126]. The root cap is the site of light perception and bending of the root is due to a larger amount of growth at the side of the root tip irradiated as compared to the shaded side [126]. The negative phototropism observed is in part influenced by auxin as auxin application can inhibit the growth, negative phototropism, and gravitropism of the rice root when exposed to light [126].

Light can also influence the growth of the primary root. It has been shown that auxin levels and polar auxin transport can mediate light-induced waving of the primary root [127]. The presence of continuous white light irradiation has also been shown to antagonize the primary root growth though crown root growth is promoted [128]. This has been found, through phytochromes mutants, to be due to phytochromes PHYB and PHYC, both of which are detected in the primary root immunochemically and correlate positively with increasing nitrogen concentration [128].

6 Conclusions and Prospects

As compared to the understanding of molecular mechanisms for the development of aerial organs in rice, our knowledge on root development and

growth, particularly at the molecular level, is largely lacking. Working on rice root is more technically challenging as compared to *Arabidopsis* root, in terms of the difficulties of live cell imaging, especially of inner cell layers as the thicker rice root has more cell layers. However, research on rice root, especially in terms of growth under environmental stresses, is crucial and important as world population and demand for food sources increase when global warming and other environmental issues are accelerating.

Fortunately, imaging technology is rapidly evolving. For example, advances in imaging have allowed for elucidating how other structures initiate from the primary root. The synchrotron X-ray computed tomography has been used as a noninvasive method to observe how aerenchyma develops from rice primary root [129]. Using a sandwich method, where a germinating rice seed is placed between two agar slabs of various osmotic concentrations, it is found that the aerenchyma is initiated from the primary root in response to osmotic stress [129]. In addition, a novel imaging and software platform has been developed to phenotype three-dimensional root traits during seedlings development [130]. This allows for high-throughput screening of large numbers of varieties, mutants, or transgenic phenotype. With expected further improvements in live cell imaging techniques for imaging rice seedlings that can detect fluorescently tagged molecular markers, the molecular mechanisms of how cells differentiate to form other structures from primary root are expected to be discovered rapidly.

Widely used "traditional" genetic methods such as QTL analysis still remain essential to discover new genes involved in root development. Recent improvements in QTL technologies, such as association mapping, specifically in the form of a genome-wide association study (GWAS) [131], shall greatly improve the speed and efficiency of QTL analysis when combined with automated imaging and high-throughput genotyping [132] and phenotyping platforms [130].

With improvements in live imaging techniques and increasing trend of genome-wide analysis and increased efficiencies of traditional methods, it is expected that more detailed mechanisms of how rice root develops can be elucidated in the near future.

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Reproductive Isolation Between *indica* and *japonica* Subspecies

21

Yidan Ouyang

Asian cultivated rice *O. sativa* is distributed worldwide with tremendous variation in genetic structure. Classification based on certain diagnostic characteristic can distinguish *O. sativa* into several subtypes, which differ in morphological, physiological, and ecological characteristics. Two main rice groups in *O. sativa*, namely *indica* and *japonica* subspecies, have been recognized based on the reproductive barriers observed. These two subspecies show strong genetic differentiation, featured by various forms of hybrid sterility. The discoveries of hybrid incompatibility genes provided opportunities to investigate the genetic mechanism of reproductive barriers between *indica* and *japonica* subspecies. The supporting evidences obtained recently also provide reasonable picture for understanding the evolutionary process of reproductive isolation in rice. In this chapter, we refocus the mechanisms of reproductive barriers between *indica* and *japonica* subspecies in three aspects, i.e., the genetic architecture, the molecular mechanisms association with rice hybrid sterility genes, as well as the evolutionary processes. But before that, we would like to introduce the population structure and geographic differentiation of the Asian cultivated rice.

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1 Population Structure and Geographic Differentiation of Cultivated Rice

Asian cultivated rice *O. sativa* was domesticated approximately 8,000–9,000 years ago from its wild progenitor *O. rufipogon* and/or *O. nivara* [1–7]. As early as approximately 2,000 years ago in the Han Dynasty, the Chinese already recognized that there were two main groups of rice, namely *hsien* and *keng*, with distinct geographical distributions. The *hsien* type is cultivated in tropical and subtropical regions of China, while the *keng* type is mostly grown in temperate regions [8, 9]. In 1928, Japanese scientists Kato et al. [10] named these two rice groups as *indica* and *japonica* based on their hybridization studies, which showed hybrids from crosses between these two rice groups were partly sterile. In the rest of this chapter, we will refer to these two groups as *indica* and *japonica* subspecies.

In a broader scale, *indica* and *japonica* subspecies have distinct growing habitats and different adaptations to the environment. *Indica* rice is distributed throughout the lowlands of tropical and subtropical regions of Asia, while *japonica* varieties are mainly dispersed in the upland areas of southern China, Southeast Asia, and Indonesia [11–13]. The *japonica* varietal groups can be further classified into temperate (*japonica*) and tropical ones (initially referred to as *javanica*) [14]. The temperate and tropical *japonica* have close genetic relationship, and

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the temperate type appears to be derived from the tropical *japonica* [14]. These two varietal groups were traditionally described by physiological, phenotypic, and agronomic characteristics, e.g., shape of the spikelets, disease resistance, cold/drought tolerance, potassium chlorate resistance, phenol reaction, plant height, and leaf color [12, 13]. It was proposed that the distinct variations observed might have arisen from the reproductive barriers between *indica* and *japonica* subspecies [10, 12].

2 Earlier Concepts of Reproductive Barriers Between *indica* and *japonica* Subspecies

Plant species are isolated by different forms of reproductive barriers. Prezygotic reproductive isolation preventing zygote formation before mating always takes place in distantly related organisms. Postzygotic reproductive barrier results in fitness aberration in hybrids, such as hybrid necrosis/weakness and hybrid sterility [15, 16]. Hybrid sterility between *indica* and *japonica* rice has been observed since the work of Kato et al. as early as 1928 [10]. Subsequent studies reported that the majority of inter-subspecific crosses showed significantly reduced spikelet fertility [12]. A diallel crosses of 21 parents representing a broad range of rice germplasm provided comprehensive data that most inter-subspecific hybrids showed partial to complete sterility [17]. Further analysis also confirmed the findings that the sterility was caused by the reproductive barrier in *indica–japonica* hybrid [18]. The *indica–japonica* hybrids encounter difficulties in reproductive development at various stages [12, 19–25], and the male and female gamete abortions contribute almost equally to the inter-subspecific hybrid sterility in typical *indica–japonica* crosses [23].

Intermediate rice varieties were also observed first by Morinaga and Kuriyama [26]. It was proposed that some varietal groups such as *Bulu* and *Aus* can produce fertile hybrids when crossed with either *indica* or *japonica* rice. The existence of such rice group was further confirmed by Ikehashi and Araki in screening a total of 74 varieties by crossing them to *indica* and *japonica* testers [27]. Such type of varieties was regarded as wide-compatibility varieties (WCVs), carrying a neutral allele (also referred to as wide-compatibility gene, WCG) at the hybrid sterility locus [27]. Despite the increasing understanding of hybrid sterility and wide-compatibility between *indica* and *japonica* subspecies, there are a series of questions in when and why reproductive isolation takes place.

3 Genetic Differentiations Between *indica* and *japonica* Result in Hybrid Sterility

Several molecular studies estimated that the divergence time between *indica* and *japonica* variety groups was approximately 0.2–0.4 million years ago [28–30]. However, the origin and evolutionary history of these two subspecies have long been debated. One hypothesis suggested that differentiation between *indica* and *japonica* occurred after the domestication of *O. sativa*, while the *japonica* rice might be derived from *indica* group [12, 31]. This model gained support from molecular evidence by microsatellites [32] and SNP data [33] and sequence comparison in domestication genes [34, 35]. Another competing hypothesis, namely independent origin model, suggests that these two rice groups might originate separately from their pre-differentiated wild ancestors [3, 4, 36–40]. This independent origin model can explain the differentiated gene pools observed in *O. rufipogon*, which was associated with distinct clades of *indica* and *japonica* varieties by phylogenetic analyses [13, 30, 36, 38, 41–44]. Evolutionary analysis in rice speciation gene *S5* also supports the result that *indica* and *japonica* differentiation already occurred in the wild rice species [45, 46]. Recently, a comprehensive map of rice genome variation revealed that *japonica* rice was first domesticated from a specific population of *O. rufipogon* around the middle area of the Pearl River in southern China. It was proposed that *indica* rice was subsequently developed from crosses between *japonica* rice and local wild rice as the initial cultivars spread into

South East and South Asia [47]. This study facilitates the understanding in origination and differentiation of *indica* and *japonica* groups, as well as the evolutionary process of rice domestication.

Indica and *japonica* subspecies differ significantly in genome structure of the population, thus resulting in divergence in phenotypic variation, molecular diversity, and adaptation. Investigations by molecular markers suggest that genetic differentiation appears to be a major source of genetic diversity in the cultivated rice gene pool [14, 18, 48–53]. Distinct inter-subspecific sequence polymorphisms were detected by genome sequencing of typical *indica* and *japonica* varieties [54, 55]. In addition, genome sequences from diverse sample of worldwide rice varieties have also identified the existence of divergent groups, showing a very strong population differentiation between *indica* and *japonica* [56–58]. Reproductive barriers are generally regarded as the by-product of linage-specific divergence attributed to accumulation of genetic differentiation. Differentiation between *indica* and *japonica* has led to genetic incompatibility preventing gene flow, and ultimately inducing reproductive isolation. The interpretations of the mechanism underlying reproductive barriers are strongly supported by the functional characterization and sequencing analysis of hybrid incompatibility genes cloned in rice recently.

4 Glimpse from the Hybrid Incompatibility Genes in Rice

Genetic analyses have identified approximately 50 loci controlling either female gamete abortion or pollen sterility (in a few cases, both) in rice [59], suggesting that reproductive isolation is the cumulative consequence of complex interaction between a large number of diverged loci. Hybrid sterility genes cloned and characterized provide confirmative data for elucidating the cellular and molecular mechanism of the reproductive barriers.

The *S5* locus identified for rice hybrid sterility conferred significant effect on embryo-sac fertility in *indica–japonica* hybrids [60] (Table 21.1). This locus was primarily mapped on chromosome 6 and further delimitated into a 40-kb fragment containing five open reading frames (*ORF1-5*) [61–68]. Map-based cloning and genetic transformation confirmed that expression of the *indica* allele *ORF5+* in a *japonica* variety caused female gamete abortion, which identified *ORF5* as the candidate for the *S5* locus [60]. The *ORF5* transcripts accumulate in the ovule tissues, suggesting that the ORF5 encoding an aspartic protease functions in megaspore formation or survival. There are only two nucleotides differences in the *indica* (*ORF5+*) and *japonica* (*ORF5-*) alleles in the coding sequence, which cause two amino acid substitutions in the central domain based on crystal structure analysis [60, 69, 70]. The wide-compatibility allele (*ORF5n*) is identified with a deletion in the signal peptide and N-terminal segment of the central domain. The destination of ORF5n protein in WCVs is thus changed into the cytoplasm, whereas in the case of ORF5+ and ORF5-, the protein will be secreted into the cell wall [60].

Further genetic analysis confirmed that *ORF3* and *ORF4*, which were predicted to encode a heat shock protein (Hsp70) and a membrane protein, respectively, were also required for the *S5-induced* hybrid sterility and segregation distortion [46] (Table 21.1). The *ORF3* in typical *japonica* varieties (*ORF3–*) has a 13-bp deletion relative to that in typical *indica* varieties (*ORF3+*), while the *indica* allele of *ORF4* (*ORF4–*) has an 11-bp deletion compared with that in typical *japonica* varieties (*ORF4+*). Thus the typical *indica* and *japonica* varieties contain the haplotype of *ORF3+ORF4–ORF5+* and *ORF3–ORF4+ ORF5–*, respectively. The *ORF*3, *ORF*4, and *ORF*5 are tightly linked, and a killer composed of ORF5+ and ORF4+ would selectively kill the female gametes without the protector of ORF3+ in inter-subspecific hybrids. It was proposed that the extracellular ORF5+ produced a molecule that was sensed by plasma membrane-localized ORF4+, which eventually triggered endoplasmic reticulum (ER) stress in ovaries. The ER stress would subsequently induce premature programmed cell death (PCD) in the developing megaspores without *ORF3+*, resulting in preferential

Table 21.1 Hybrid incompatibility genes causing postzygotic reproductive isolation in rice

Crosses	Loci	Gene structure	Alleles	Hybrid phenotype	Genetics	References
O. sativa ssp. *indica* × ssp. *japonica*	*S5 ORF3* *ORF4* *ORF5*	Heat shock protein Hsp70 Membrane protein Aspartic protease	*ORF3+/ORF3–* *ORF4+/ORF4–* *ORF5+/ORF5–/ORF5n*	Embryo-sac sterility and preferential abortion of female gametes with *ORF3–*	*ORF5+* in combination with *ORF4+* can selectively kill the female gametes without *ORF3+*	[46, 60]
O. sativa ssp. *indica* × ssp. *japonica*	*Sa SaF* *SaM*	F-box protein Small ubiquitin-like modifier E3 ligase-like protein	SaF^{+}/SaF^{-} SaM^{+}/SaM^{-}	Pollen sterility and preferential abortion of male gametes with SaM^{-}	Dominant alleles of SaM^{+} and SaF^{+} can selectively eliminate the male gametes with SaM^{-}	[71]
O. sativa ssp. *indica* × ssp. *japonica*	*DPL1* *DPL2*	Duplicated genes encoding plant-specific small proteins	$DPL1\text{-}N^{+}/DPL1\text{-}K^{-}$ $DPL2\text{-}K^{+}/DPL2\text{-}N^{-}$	Pollen failed to germinate	Recessive alleles at both loci in heterozygotes can cause incompatibility One functional *DPL* is required for pollen development	[72]
O. sativa × *O. glumaepatula*	*S27* *S28*	Duplicated genes encoding mitochondrial ribosomal protein L27	$S27\text{-}T65^{+}/S27\text{-}glum^{s}$ $S28\text{-}glum^{+}/S28\text{-}T65^{s}$	Pollen sterility	Recessive alleles at both loci in heterozygotes can cause incompatibility One functional mtRPL27 is required for pollen development	[73]

abortion of female gametes and segregation distortion in the offspring. Such ER stress would be resolved by ORF3+, thus producing normal female gametes by preventing the premature PCD.

The other two incompatibility genes cloned in *indica–japonica* hybrids were identified to cause male sterility (Table 21.1). The *Sa* locus was also isolated by map-based cloning approach [71] (Table 21.1). The *SaM* and *SaF* genes at this locus generate a rather precise system in controlling the development of the male gametes in hybrids. These two adjacent genes, encoding a small ubiquitin-like modifier E3 ligase-like protein and an F-box protein, respectively, are expressed constitutively. Most *indica* varieties carry the haplotype of $SaM^{+}SaF^{+}$, whereas all *japonica* cultivars investigated possess $SaM^{-}SaF^{-}$. The intermediate haplotype of $SaM^{+}SaF^{-}$ is compatible with both $SaM^{-}SaF^{-}$ and $SaM^{+}SaF^{+}$ due to the absence of SaF^{+} or SaM^{-}, which might be referred to as the WCG at *Sa* locus. The genetic analysis combined with molecular characterization suggested that gametes carrying SaM^{-} were selectively eliminated, for the reason that the specific complex of SaF^{+}-SaM^{-} might interact negatively with SaM^{+} in heterozygotes. Therefore, hybrid male sterility would occur due to the allele-specific abortion of gametes, thus resulting in segregation distortion [71].

Whole genome survey of two-way interacting loci detected one reproducible interaction on rice chromosomes 1 and 6. Combined with map-based cloning, paralogous genes of *DPL1* and *DPL2* encoding plant-specific small proteins were identified to cause genetic incompatibility in *indica–japonica* hybrids [72] (Table 21.1). The functional alleles of $DPL1\text{-}N^{+}$ and $DPL2\text{-}K^{+}$ exhibit abundant expression level in mature anther. However, the expression level of the defective $DPL1\text{-}K^{-}$ is undetectable, and the protein of $DPL2\text{-}N^{-}$ is absence. Therefore, pollen grains carrying both of the loss-of-function alleles together fail to germinate. Such impaired pollen transmission could be rescued by either $DPL1\text{-}N^{+}$ or $DPL2\text{-}K^{+}$, suggesting that at least one functional allele of *DPLs* is essential for pollen germination [72].

Another pair of duplicated genes, namely *S27* and *S28*, was identified as conditioning hybrid pollen sterility between the cultivated rice *O. sativa* and its wild relative *O. glumaepatula* [73] (Table 21.1). *S27* and *S28* encode mitochondrial ribosomal protein L27 and function in a gametophytic manner, thus pollen grains carrying a set of sterile alleles, $S27\text{-}glum^{s}$ and $S28\text{-}T65^{s}$, would be sterile. However, either one of the fertile alleles, $S27\text{-}T65^{+}$ or $S28\text{-}glum^{+}$, has the ability to rescue the sterile phenotype in heterozygotes. Transformation and expression analysis confirmed that the sterile alleles of *S27* and *S28* were loss-of-function, due to the absence of the duplicated segment in $S27\text{-}glum^{s}$ and failure of expression in $S28\text{-}T65^{s}$. It was inferred that mtRPL27 was deficient in protein synthesis in mitochondria, thus impairing respiratory activity and resulting in pollen sterility [73].

In conclusion, these hybrid sterility genes seem to play different roles in development and biological processes in rice with diverse functions, encoding various types of proteins from enzymes to structural proteins, being either plant-specific or conserved in eukaryotes. We should notice that the hybrid sterility genes are extremely essential in some cases, such that one functional copy is required in the gamete development [72, 73]. However, in other case the speciation gene might not play important roles in rice growth or reproduction, as the varieties with nonfunctional allele of *ORF5* at *S5* locus show normal phenotype [60]. Despite the complexity of molecular mechanism, these hybrid sterility genes share common features at the genetic level.

5 Genetic Basis of Reproductive Isolation Between *indica* and *japonica* Rice

5.1 The Classical Dobzhansky–Muller Model

It is important to describe a more general framework of how hybrid incompatibility forms. The classical Dobzhansky–Muller model proposed a

fundamental conception for understanding the genetic essence of reproductive isolation [74]. This model explains that the postzygotic reproductive isolation results from deleterious interactions between two or more functionally diverged genes in the respective hybridizing species. In this model, independent mutations occurred and accumulated in each evolved population when the ancestral species diverged. Both of these mutations are neutral within the populations in which they arose. However, the incompatible loci interact negatively and cause deleterious effects when coming together in hybrids.

The classical Dobzhansky–Muller model emphasizes the role of epistasis and recessivity. Different genetic materials are required for mapping hybrid dominant and recessive incompatibility genes. The deleterious interaction between corresponding genes occurs in the F_1 generation when both loci are heterozygous if the alleles were dominant. When the hybrid incompatibility genes are recessive, F_2 or backcross progeny carrying homozygous loci are needed to detect the effect of the loci. The Dobzhansky–Muller model provides a simple explanation of the evolution of intrinsic postzygotic isolation, which plays an important part in both theoretical and experimental studies of hybrid sterility.

5.2 Genetic Models Proposed for *indica–japonica* Hybrid Sterility

The genetic basis of hybrid sterility was investigated by many researchers, while several models have been proposed in rice. A duplicate gametic lethal model suggested that two independent loci affected the development of the gametes [75, 76] (Fig. 21.1). It was proposed that gametes carrying the recessive alleles at both loci were aborted, whereas gametes with at least one dominant allele were fertile [75, 76]. In this model, the deleterious interaction occurred between two recessive alleles in the hybrids, which can be regarded as a special case of the Dobzhansky–Muller model. Two pairs of hybrid sterility genes in rice, *S27* and *S28*, as well as *DPL1* and *DPL2*, are compatible with this model [72, 73] (Fig. 21.1). In addition, hybrid sterility genes other than rice also fit well with this model [77–80], thus suggesting that such genetic architecture exists widely in eukaryotes.

Another influential model for *indica–japonica* hybrid sterility was the one-locus sporogametophytic interaction model proposed by Kitamura [81] (Fig. 21.1). This model assumed that the allelic interaction in a heterozygous genotype selectively eliminated the gametes carrying the *japonica* allele. In this system, varieties carrying neutral alleles can produce fertile hybrids with either *indica* or *japonica* subspecies. This system was further developed as the allelic interaction model by Ikehashi and Araki [27]. They examined the hybrid sterility at *S5* locus and suggested that there were three alleles at the *S5* locus: an *indica* allele (*S5*-i), a *japonica* allele (*S5*-j), and a neutral allele (*S5*-n) (WCG) [27]. Sterility occurs only when the plants have *S5*-i and *S5*-j alleles simultaneously, whereas such reproductive barrier can be overcome by hybridization with the plants carrying *S5*-n. The behavior of many identified hybrid sterility loci in rice supports this model as revealed by general genetic analyses [19, 68, 82, 83]. However, it should note that hybrid sterility in this model results from the selective abortion of gametes with a given allele. Such preferential transmission of different gametes into the progeny, namely segregation distortion, can also be regarded as a possible interpretation of the standard Dobzhansky–Muller model in which deleterious interactions occur between sporophytic and gametophytic alleles [46]. Strong supporting evidences have been found for both models mentioned above, such that hybrid sterility is induced by complex interaction between at least two components [71–73, 84–86]. Recent understandings have proposed three evolutionary genetic models to depict the processes for installing the hybrid incompatibility systems [87]. The parallel divergence model suggests that deleterious interaction between two parallelly diverged alleles of the duplicated

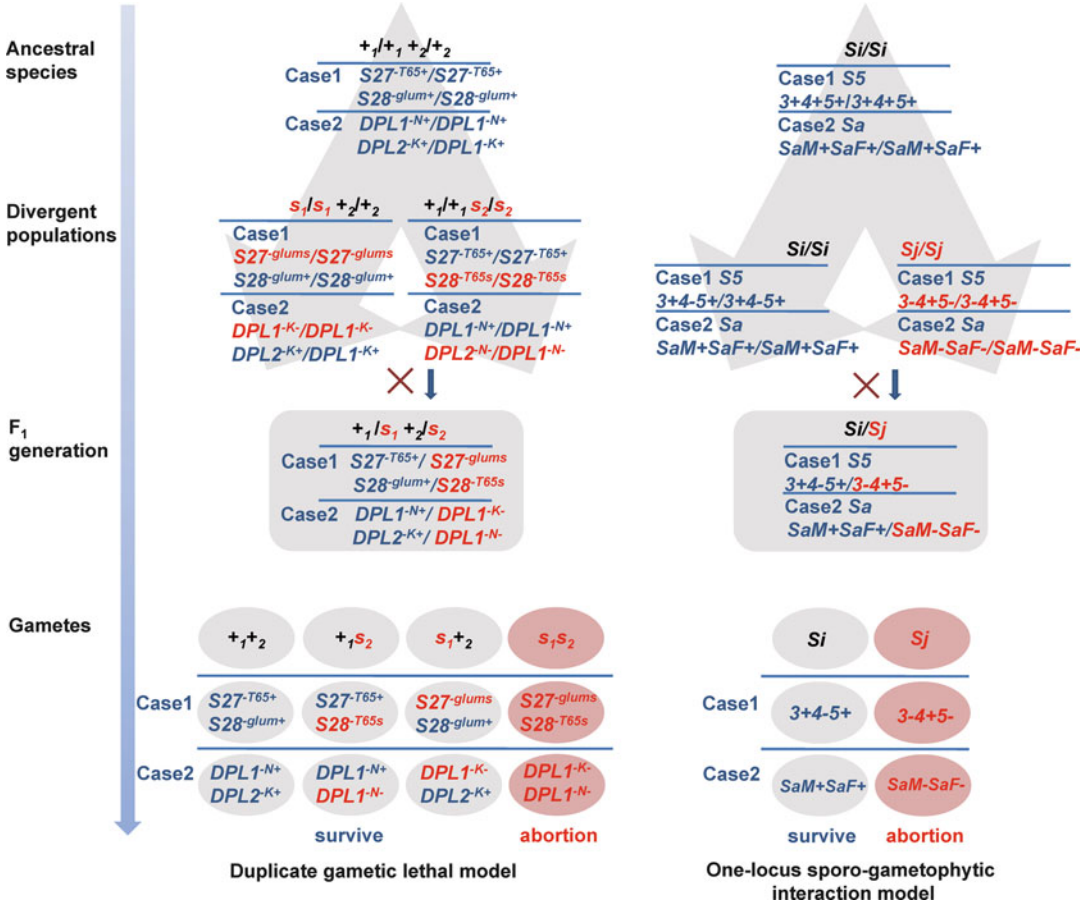

Fig. 21.1 Genetic models proposed for *indica-japonica* hybrids. In the duplicate gametic lethal model (*left*), the ancestral species contains the genotype of +1/+1 +2/+2, which has functionally diverged into *s1*/*s1* +2/+2 and +1/+1 *s2*/*s2* in the respective hybridizing species. The gametes carrying the recessive alleles at both loci, namely *s1s2*, are aborted in heterozygous background, whereas gametes with at least one dominant allele are fertile. In the one-locus sporo-gametophytic interaction model (*right*), those gametes having *Sj* allele are selectively aborted in a heterozygous genotype

loci can cause hybrid incompatibility. The sequential divergence model emphasizes that mutations of two linked loci occur sequentially in one lineage, and negative interaction would occur between the ancestral and nascent alleles of different genes. The parallel-sequential divergence model is more complex. This model is exemplified by a killer–protector system with three tightly linked loci, and incompatible interactions arise from mutations in two steps. It should be noted that both the duplicate gametic lethal model and the one-locus sporo-gametophytic interaction model can be well placed into the perspective of the evolutionary genetic models here [87].

6 Evolution of Reproductive Isolation Between *indica* and *japonica* Is a Continuous and Long-Lasting Process

The evolutionary history of reproductive isolation between *indica* and *japonica* subspecies is also a long-lasting process, and the emergence of rice hybrid sterility genes has occurred continuously (Fig. 21.2). The different allelic groups at *S5* locus have already existed in both cultivated rice and its wild relatives, suggesting that the divergence occurred before the speciation of *O. sativa*, *O. nivara,* and *O. rufipogon* [45, 46].

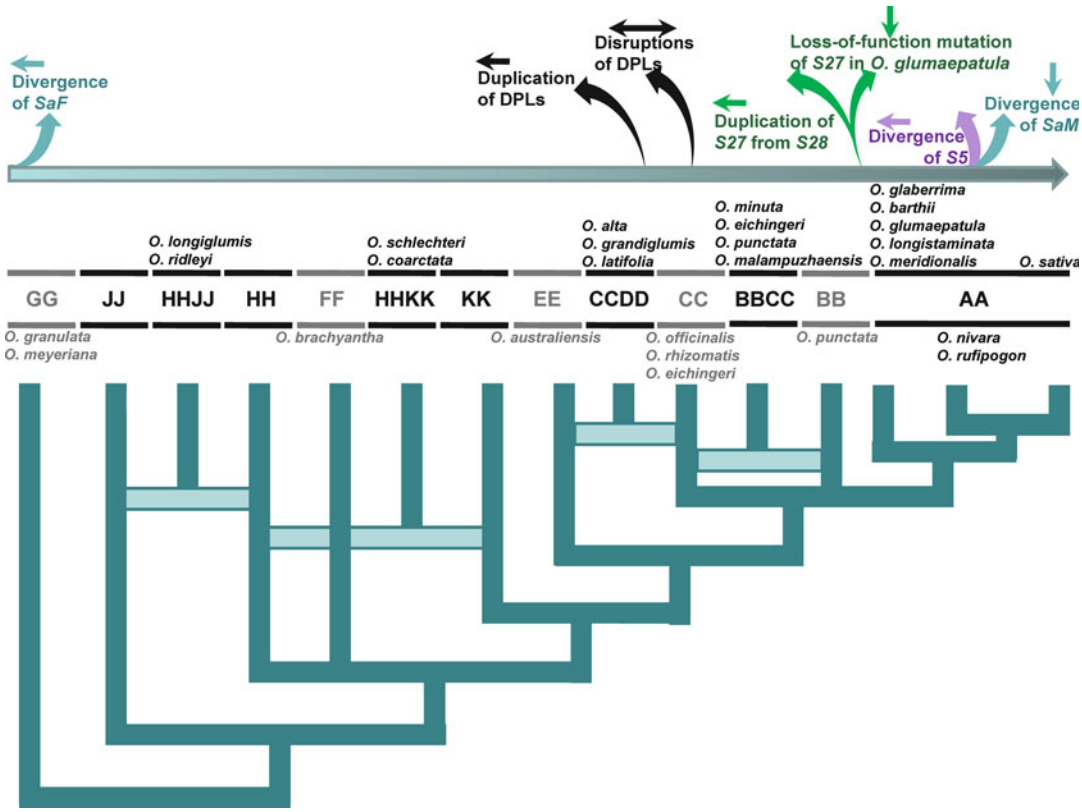

Fig. 21.2 Schematic illustration of the evolutionary history of the hybrid incompatibility genes in rice. The phylogeny of rice genome types and the differentiation events of the speciation genes are determined according to the references published [46, 88–91]. The horizontal arrows indicate that the divergence or duplication occurred before the time point. The vertical arrows indicate that the variations most likely occurred in the time as suggested

The variation in *SaF* occurred before the split of most of the *Oryza* species, whereas the mutation in *SaM* most likely arose in *O. rufipogon* [71]. This result suggested that the two components of the *Sa* system originated sequentially at different time [71]. Sequence and expression analysis showed that duplication events of *DPLs* occurred at least before the speciation of *O. officinalis* from the common ancestor of rice [72]. In addtion, both DPLs are functional in 16 accessions, which indicated that *DPL* genes might not induce reproductive barriers among these species [72]. However, disruptions of *DPL* genes exist in several rice accessions, suggesting that *DPL*s might induce reproductive barriers among these species [71]. The presence of *S27* and *S28* was also determined in 172 rice accessions of the eight AA genome species. The results showed that *S27* was derived from *S28* and duplication of *S27* occurred before the divergence of the rice AA genome species, except *O. glumaepatula* [73, 88]. Besides, *S27+* existed in seven species except *O. glumaepatula* [73, 89]. All these results depict a clear picture that hybrid sterility genes emerged and diverged in a wide range of time period, inducing reproductive barriers in rice by interactive effects (Fig. 21.2) [89–92].

7 Approaches for Identifying Hybrid Incompatibility Genes in Rice

7.1 Map-Based Cloning

Map-based cloning might be regarded as the most traditional method for cloning hybrid incompatibility genes. Map-based cloning can

discover different types of reproductive barriers. This method can be used if the F_1 hybrids are sterile. It is also applicable for determining genes that have segregation distortion in F_2 or backcross populations. Hybrid incompatibility genes that act in a gametophytic mode or lead to zygotic lethality would lead to preferential transmission of different gametes into the progeny. Such deviations from Mendelian ratios can be detected easily either in the backcross or F_2 populations.

There are also some technical constraints in map-based cloning method. For instance, it is always necessary to develop near-isogenic lines (NILs) to eliminate the influence on fertility by the minor loci from the background. NILs are constructed by backcrossing the candidate chromosomal segments from one subspecies into the genetic background of another. Thus, the progressing of genetic mapping is slow and time-consuming. Besides, large numbers of recombinant plants from the F_1 or F_2 individuals are needed, in order to delimitate the candidate genes to a region narrow enough. Despite these limitations, all the four rice hybrid incompatibility genes identified were cloned by map-based cloning [46, 60, 71–73], suggesting that it remains to be an efficient way in future studies.

7.2 Complementary Approaches for Genetic Mapping

Genetic incompatibility in *indica–japonica* hybrids might be caused by Dobzhansky–Muller interactions. Therefore, whole genome survey of two-way interacting loci acting within the gametophyte or zygote from the inter-subspecific cross is effective for identifying the hybrid incompatibility genes. Although the effect of possible interactions between loci is always very little or no significant [23], this method seems to be efficient in screening the candidates successfully, with the detection of *DPL1* and *DPL2* [72]. Genome-wide surveys of deviations from expected Mendelian segregation ratios in inter-population crosses have also provided valuable clues to identify candidates inducing reproductive barriers [93–96]. Such method is effective for detecting and characterizing reproductive barrier regulators acting in the male/female gametophyte or the zygote. Regulatory genetic pathways can provide a plausible source of the epistatic variation that has been involved in postzygotic reproductive isolation [97]. Transcriptional profiling revealing expression differences between parents and hybrids can thus determine possible changes that are associated with hybrid incompatibility [98]. All these methods might accelerate the molecular cloning of the hybrid incompatibility genes.

8 Conclusions and Perspective

Knowledge of the genetic and evolutionary mechanism involved in the reproductive barrier between *indica* and *japonica* subspecies facilitates the understanding in plant speciation. The coexistence of *indica* and *japonica* rice groups and the WCVs and the large number of loci identified so far provide an excellent model system for understanding the evolutionary processes of reproductive isolation and speciation.

The reproductive barrier between *indica* and *japonica* subspecies has became established through the gradual accumulation of mutations at multiple loci during the evolution. When the two rice groups have accumulated enough divergence, they become genetically differentiated and cause reproductive isolation. Such genetic differentiation between *indica* and *japonica* has promoted genetic diversification and enabled the wide adaptation of cultivated rice throughout the world, which enriches the rice gene pool.

Conversely, the WCVs act as a promoting factor for the interspecific or inter-subspecific gene flow, which increase the frequency of hybridization between separate populations. Thus the WCGs provide the potential exchange of genes and suppress the differentiation between the reproductively isolated rice groups. Therefore, it is highly interesting to illuminate the evolutionary mechanisms and complex gene interactions involved in this counter-acting dynamic process of reproductive isolation and speciation.

Rice plays a critical role in world agriculture. The hybrid sterility and wide-compatibility system has significant implications for utilizing the

inter-subspecific heterosis in rice, as the WCVs are able to break the reproductive barrier between *indica* and *japonica* subspecies. WCGs can be introgressed into the elite parents whose hybrids show high yield. Other strategies such as breeding "*indica*-compatible *japonica* lines" or suppressing the expression of hybrid sterility genes might also facilitate rice genetic improvement [99]. The efforts in efficient utilization of the *indica–japonica* heterosis would depend on identification and characterization of more hybrid sterility loci as well as further understanding in molecular mechanism of reproductive isolation between *indica* and *japonica* subspecies.

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Genomics-Based Breeding Technology

22

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1 Introduction

Plant breeding technologies have contributed a great deal to the elevated yield levels of crop varieties. The increased grain output in the limited arable land has so far successfully met human demand for food. But the exponential growth of world population is still going on. By 2050, world population will potentially reach nine billion and requires 70–100 % increase in food production relative to current levels [1]. There is a further demand for more efficient breeding technologies to sustain the increase of grain production and at the same time to conserve our environment by limiting the adverse impact of agriculture on the global ecosystem. Genomics-based breeding as a recently emerged breeding methodology, which applies high-throughput molecular marker platforms to genome-wide screens for preferred traits and optimized gene background, will mark a new era in plant breeding history following the hybridization breeding to meet our needs for both adequate food supply and living-friendly environment.

Significant progress has been achieved in rice (*Oryza sativa* L.) functional genomics since the completion of the international rice genome sequencing project [2], which offers tremendous opportunities for breeders to improve this important crop via genomics-based breeding. As more and more genome sequences of different rice varieties are becoming publicly available, and powerful molecular marker assay platforms have been developed, genomics-based breeding will become a common practice in the breeders' community. This chapter describes several molecular marker platforms at various levels of throughputs that may help enhance rice breeding efficiency.

2 Molecular Marker Development and Assay Platforms

The development and subsequent uses of molecular markers in the detection of ideal genotypes is one of the most significant technological

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advances in plant breeding. Indeed, molecular markers have made possible the identification of quantitative trait loci (QTL) and the establishment of new breeding strategies based on genotypic selection, where individuals are selected on the basis of genotypes instead of phenotypes. Various types of molecular markers have been developed to meet the requirements in the genotyping of various breeding materials. The first generation of DNA-based molecular markers is restriction fragment length polymorphism (RFLP) assayed by Southern blot hybridization. Its application to genetic studies resulted in the first genetic linkage map of rice and cloning of many important genes. Since the invention of PCR technology and its routine utilization in genetic studies and breeding, DNA amplification-related molecular markers have been more widely used and thus considered as the second generation of DNA-based molecular markers. The third-generation DNA markers were recognized as the application of DNA chip technology to DNA polymorphism assays. Instead of blotting genome DNA onto a nylon membrane and hybridizing a single probe to the blot, DNA probes are arrayed on a microchip to facilitate a quick analysis of a large number of molecular markers in parallel. Recent advances in DNA sequencing technologies have opened another avenue towards building a high-throughput platform for gene identification. Genotyping by direct sequencing has largely changed the strategies in genetic studies and removed the bottleneck of limited number of available molecular markers in the study of interest traits, chromosome regions, or genomes. It also removes the tedious step of polymorphism tests in synthetic populations. Direct sequencing has been widely used in genome-wide association studies and many valuable genes or QTL have been identified in the last several years. The different categories of molecular markers have their own values in genetic studies and breeding programs. Their complementary features would help plant breeders optimize molecular marker assay platforms with low-cost, high-reproducibility, and desired throughput.

2.1 Electrophoresis-Based High-Throughput Marker Assay Platforms

AFLP and other PCR-based markers generally show DNA fragment length polymorphisms that can be easily resolved using capillary columns, such as Sanger sequencers (e.g., ABI 3730xl sequencer). In this way, throughput of electrophoresis-based marker assay is largely elevated. On this assay platform, polymorphic DNA fragments need to be labeled with fluorescent dyes that are usually attached to PCR primers in order to be detected by a laser sensor in the equipment. Any type of molecular marker that shows fragment length polymorphisms, such as CAPS, InDel, AFLP, SSR, and ISSR, can be assayed using Sanger sequencers. Similar capillary equipments specifically designed for molecular marker analysis are also available on the market, such as microchip capillary electrophoresis [3].

Of the fragment length polymorphic markers, SSRs are most widely used in rice marker-assisted selection (MAS) due to their multi-allele features and assay flexibility. Either gel electrophoresis or capillary sequencer can be used to detect SSR polymorphisms. They are convenient tools available to breeders for MAS of target traits. In order to increase throughput and reduce labor intensity in SSR assays, Sanger sequencers have been employed to resolve SSR polymorphisms. There are a few advantages of using capillary sequencer to detect fluorescence-labeled SSR markers [4]: (1) It largely increases the throughput using a multiplex method that assays a group of SSR markers at a time by detecting various sizes of SSR fragments with the corresponding fluorescence-labeled primers, (2) it significantly increases the accuracy of allele-calling via an internal size standard in each lane, and (3) the automated marker detecting instruments and allele-calling software dramatically increase the efficiency and reduce labor intensity.

In rice, Blair et al. organized 27 fluorescence-labeled SSR markers into four multiplex panels for diversity analysis of *Oryza* species [5].

Coburn et al. assembled 159 fluorescence-labeled SSR markers into 21 multiplex panels for semiautomated genotyping in rice, which offered an even coverage of the 12 chromosomes [4]. In order to evaluate the levels of genetic diversity within Basmati rice varieties and thus assess genetic relationships among the accessions, Jain et al. combined 30 fluorescence-labeled SSRs into four multiplex panels to fingerprint 69 diverse accessions [6]. A total of 235 alleles were detected at the 30 SSR loci, and the number of alleles per locus ranged from 3 to 22, with an average of 7.8 [6]. Garris et al. investigated the genetic diversity and structure of 234 accessions of rice using 169 nuclear SSRs and two chloroplast loci [7]. Pessoa-Filho et al. studied 548 accessions of traditional upland rice landraces using three SSR multiplex panels of 16 loci with a single PCR reaction for each panel [8]. The highest throughput of DNA fragment analysis so far is provided by ABI 3730xl DNA analyzer, which can analyze 96 lanes of multiplex SSR markers in a single run within less than 1 h. Conserved flanking primer sequences rich in allele numbers and automated high-throughput SSR assay platforms are the keys for a successful utilization of SSR markers in molecular breeding programs, especially in the target trait selection.

2.2 Array-Based High-Throughput Marker Assay Platforms

2.2.1 Diversity Array Technology

Diversity array technology (DArT) markers were developed by Jaccoud et al. [9] who successfully apply the microarray platform to the analysis of DNA polymorphisms. In this method, representative DNA fragments of an organism or a population of organisms are selected and arrayed on a chip to be used as DNA probes to detect the presence/absence or signal intensity variation of the assayed DNA samples when they are hybridized to the chip. Typically, the representative DNA fragments are selected from genetic diversity panels that allow genetic fingerprinting of any organism or group of organisms belonging to the gene pool from which the panel was developed. DArT marker assays do not need electrophoresis and can be performed in a high-throughput manner. Genome sequence information is not required for the development of DArT marker assays, so DArT markers are especially suitable for application in crops without available genome sequences, such as wheat. However, DArT markers can be subsequently sequenced in order to anchor them to a reference sequenced genome.

DArT markers have been widely used in germplasm fingerprinting and genetic diversity or evolution studies. A DArT microarray was developed in rice by using a diversity panels of 22 cultivated rice varieties plus two controls (CO39 and LTH) representing typical *Oryza indica* and *O. japonica* subspecies, respectively [10]. The founder of the DArT technology set up a company named Diversity Arrays Technology Pty. Ltd. (http://www.diversityarrays.com/), which delivers affordable products and services in genome profiling, genetic analysis, and molecular breeding for tens of species, including barley and wheat. However, the process of constructing representative genomic DNA library is complex and time-consuming before DArT assay, which has limited its utilization especially in those species such as rice with genome completely sequenced.

2.2.2 Restriction Site-Associated DNA

Restriction site-associated DNA (RAD) markers are a genome-wide representation of every site of a particular restriction enzyme by short DNA tags. DNA sequence variations that disrupt restriction sites allow RAD tags to serve as genetic markers throughout a genome. Miller et al. demonstrated the applicability of RAD markers for both individual and bulk-segregant genotyping [11, 12]. They developed a RAD marker microarray resource that allows high-throughput, high-resolution genotyping. RAD markers can be identified and scored by detecting differential hybridization patterns of RAD tags on a microarray. In RAD markers assays, the two samples are separately digested with a particular restriction enzyme and then ligated to biotinylated linkers. The DNA is randomly sheared leaving only the fragments that were

directly flanking a restriction site attached to biotin linkers. These fragments are purified using streptavidin beads and released by digestion at the original restriction site. Hybridization of purified RAD tags to a DNA microarray results in differential signal patterns. As compared to RFLP, CAPS markers that only represent a small subset of available restriction sites for any particular enzyme, RAD allows for nearly every restriction site of a particular enzyme to be screened in parallel.

2.2.3 Single Feature Polymorphism

Single feature polymorphism (SFP) markers are high-throughput markers based on the detection of differential hybridization signals of various alleles to DNA probes arrayed on a microchip. Once labeled, genomic DNA or cDNA samples are hybridized to a microarray chip; the fluorescence signals at each spot where a probe is located will reveal the perfectly matched or mismatched alleles. Any sequence variation, e.g., single-nucleotide polymorphism (SNPs) or InDels in the genomic sequences of the tested samples, will be called by the corresponding probes via the reduced hybridization signals. SFPs can be used as markers to genotype segregation populations or to fingerprint germplasms. In particular, Affymetrix microarrays, composed of hundreds of thousands or millions of probes each containing 25 oligonucleotides that are sensitive to sequence variations, are efficient and convenient to detect very large numbers of polymorphisms in a single experiment [13, 14]. SFP markers were first used for QTL mapping in yeast in 1998 [15], and later were applied in *Arabidopsis* [16–21], rice [22–24], barley [25, 26], wheat [27, 28], tomato [29], and other organisms. However, the disadvantage of the platform resides in the difficulty to control false-positive SFPs when using genomic DNA samples or to distinguish true SFPs from gene expression markers when using cDNA samples. Nevertheless, SFP markers can be used to study genetic control of gene expression patterns when cDNA samples are hybridized to the microarray chip.

2.2.4 Single-Nucleotide Polymorphism Markers and Assays

SNPs are defined as single-base pair variations in genomic DNA, at which different sequence alternatives (alleles) exist in either normal or mutated individuals in the studied population(s) [30]. Synonymous SNPs do not change the coded amino acid and thus can be enriched in genome without selection pressure. Therefore, SNPs are the most abundant and ubiquitous type of polymorphisms in all organisms. The abundance of these polymorphisms in plant genomes makes the SNP marker system an attractive tool for gene/QTL mapping, marker-assisted breeding, and map-based cloning [31–33]. Utilization of SNP markers in genetic studies has significantly increased the density of the linkage maps in various organisms and facilitates fine-mapping of candidate genes/QTL or determination of *haplotypes* associated with traits of interest. Some asynonymous SNPs gain fitness advantages and thus can be used a functional marker for the direct selection of target traits. SNPs are highly stable, most frequently bi-allelic in populations, and their allele frequencies can be estimated easily in any population. The rapidly increasing DNA sequence data of rice are becoming publicly available, which ensures SNP-based markers to be more commonly and widely used than any other types. SNPs are the most abundant type of polymorphism in plant genomes. Recent studies identified approximate five SNPs per kb across 20 *Arabidopsis* accessions [34] and nine SNPs per kb across 517 re-sequenced rice varieties [35]. And SNPs are usually bi-allelic and present a bias towards transitions over transversions [36]. Thus, SNPs are now overtaking SSRs due to the higher efficiency and lower cost of genotyping achieved by using high-throughput SNP assay platforms.

Several methods have been developed for SNP assays. Traditionally, three strategies are commonly used in the detection of SNPs: (1) AS-PCR (allele-specific PCR), using single locus-specific primer and two allele-specific primers with each containing the allele-specific nucleotide at the 3′

end to amplify the target SNP fragments [37]; (2) converting SNPs to CAPS/dCAPS markers using online software dCAPS Finder [38]; and (3) direct sequencing the PCR products containing the SNP sites using Sanger sequencing method. However, more efficient chip-based platforms and next-generation sequencing technologies are gradually replacing these methods. Chip-based DNA arrays of various levels of SNP probe density and assay throughput can largely meet the genotyping requirement of different breeding materials.

Low- and Medium-Density SNP Assays

A custom-designed low-density DNA chip named GoldenGate platform has been widely used to detect 96-, 192-, 384- or up to 1536-plex SNPs on each array in combination with Veracode technology and BeadXpress Reader technology (http://www.illumina.com). SNPs and their flanking sequences are used to design locus- and allele-specific primers. Allele-related DNA fragments are amplified via allele-specific primer extension, ligation, and subsequent universal PCR reactions. Meanwhile, locus-specific probes are synthesized and laid out on a microchip, and each probe is attached to an address sequence to report the corresponding locus information. When allele-labeled PCR fragments are hybridized to the corresponding locus probes on the DNA chip, the information on the presence/absence of any alleles is caught by the detection of colors of fluorescence dyes attached to each allele-specific fragment. The collection of SNP primers is termed as an oligonucleotide pool, or an OPA (oligo pool assay). The main advantages of GoldenGate assay platform are (1) its high reliability and (2) its little technical adjustment requirement once the array design and assay optimization are setup. This platform can rapidly assay thousands of individual samples within a short time window and is relatively inexpensive as compared to other marker technologies. Recently, BeadXpress OPAs with 384 SNPs have been developed in crops and used for DNA fingerprinting, quantitative trait locus (QTL) mapping, MAS, and development of specialized genetic stocks [39–42]. Low-density SNP array platforms are useful to breeders in the survey of genetic diversity of germplasm collections and genotyping early generation of breeding materials because of their high-throughput capacity in sample processing, although the genetic information of a specific line or population obtained from a low-density SNP array platform is limited.

Medium-density SNP arrays are better balanced between sample processing throughput and genetic resolution of the markers as compared to low-density ones. A 1536-SNP GoldenGate array was designed to detect polymorphism within and between the five major subpopulations of *O. sativa* [43]. In another study, a set of 2,688 SNPs were used to genotype 151 Japanese rice cultivars that were released over the last 150 years [44]. Recently, Fasong Zhou and colleagues have designed a medium-density rice SNP chip (RICE6K) on Illumina's Infinium platform [45]. Different from BeadXpress and GoldenGate platforms, the Infinium platform detects SNP alleles by adding a fluorescence-labeled allele-specific nucleotide via single-base extension and subsequent detection of the fluorescence color. It eliminates the ligation step and reliability appears to be technically high. The RICE6K chip contains about 6,000 bead types which represent 5,636 SNPs, about 4,500 of them being of high quality and reliably detectable in assays [45]. The array design was based on the information from re-sequencing 520 genetically diverse rice germplasm accessions [35]. Over four million SNPs were obtained from the dataset. About one million SNPs with good flanking sequences were used for the design of RICE6K. Additionally, 80 SNPs/InDels representing more than 30 functional genes were selected for probe synthesis. The SNPs on the RICE6K array are evenly distributed on the rice genomes and there are on average 800 and 1,000 high-quality polymorphic markers within the *japonica* and *indica* accessions, respectively, and 2,600 between subspecies. The validation tests for RICE6K showed that the array is efficient for rice seed authenticity check, genetic background selection of early generation breeding materials, and germplasm fingerprinting [45].

Apart from Illumina's SNP chip platforms, many other array-based molecular marker technologies were also developed for massive and rapid SNP genotyping. These include GenomeLab SNPstream genotyping system (Beckman Coulter, https://www.beckmancoulter.com/) [46] and TaqMan OpenArray genotyping system (Applied Biosystems, http://www.appliedbiosystems.com/). SNPstream genotyping system uses multiplexed PCR in conjunction with tag array and single-base extension genotyping technology. Twelve or 48 multiplex PCR are performed in each well of a 384-well format on a glass-bottomed plate, and 384 samples for either 12 or 48 SNPs are genotyped per array [46]. This genotyping system has already been used for genotyping human blood group antigens [47, 48]. Using this system, Meirmans et al. developed a set of SNP markers that allows the differentiation between five commercially important species of poplar [49]. TaqMan OpenArray genotyping system consists of TaqMan genotyping assays and OpenArray technology. TaqMan genotyping assays use two allele-specific minor groove binder (MGB) probes and two primers for accurate genotyping calling [50], whereas OpenArray technology uses nanofilter fluidics for massively parallel analysis of large samples at lower costs per data point. This system provides an accurate, reproducible, cost-efficient, and labor-saving genotyping platform and expands the range of TaqMan technology usage in breeding programs [51–55].

High-Resolution SNP Assays

For genetic background selection or whole-genome examination, plant breeders need high-resolution genotyping platforms that can assay a large number of SNP markers at relatively lower cost. High-density Affymetrix SNP chip and Illumina Infinium SNP chip provide such platforms for plant breeders to perform whole-genome selection.

Two high-resolution Affymetrix custom arrays were designed in rice. One consists of ~44,000 SNPs (hereafter the 44K array) and the other consists of ~1 million SNPs (1 M array) [40]. Both have been used to assay genome-wide patterns of genetic variations in worldwide collections of wild and cultivated rice accessions. These high-resolution SNP detection platforms are more economically effective than the lower resolution assays in terms of cost per data point and can rapidly and accurately generate a large dataset of genetic diversity information on thousands of lines. The 44K array provides approximately one SNP every 10 kb, which is expected to be sufficient for genome-wide association mapping in rice. The 1 M array provides approximately one SNP every ~500 bp (of single copy DNA), with an expected 1–5 SNPs in every annotated, single copy gene in the rice genome, depending on the size and sequence complexity of the gene. These higher resolution tools allow rice breeders and geneticists to characterize natural variation across the rice genome at a reasonable cost that invites large-scale analysis. A genome-wide association study was performed recently based on the 44K SNP array across 433 diverse accessions of *O. sativa* collected from 82 countries that were phenotyped for 34 traits, and identified dozens of common variants related to complex traits [56].

Another high-resolution microarray SNP assay based on Illumina's Infinium platform has been recently developed in Maize, named MaizeSNP50 [57]. The SNP probes were selected based on the analysis of more than 800,000 SNPs and preferentially located in genes and evenly distributed across the genome. A set of maize germplasm including North American and European inbred lines, parent/F_1 combinations, and distantly related teosinte material were used to test the array. A total of 49,585 good-quality markers with 2/3 located within 17,520 different genes and 1/3 outside genes can be detected in the assay. A total of 20,913 and 14,524 polymorphic SNP markers were identified in two intermated maize recombinant inbred line populations—IBM (B736Mo17) and LHRF (F26F252)—and put on linkage maps, respectively [57]. A high-density rice Infinium chip that consists of ~50K SNPs has been also developed (Zhou et al., unpublished data). One of the biggest advantages of the high-density SNP chip is the wide range of its applications. It can guarantee

for each assay that there are enough polymorphic SNP markers evenly covering the whole genome between any two germplasm accessions. Therefore it is feasible to establish a common SNP platform for the entire rice community.

Both low- and high-resolution SNP assay platforms will remain valuable for years in genomics-based breeding. Low-resolution SNP array are much more flexible than a genome-wide deep re-sequencing, for the purpose of foreground selections on specific regions of interest. For medium- or high-resolution SNP arrays, it is more economical to design a fixed array and to apply it in genotyping of thousands of breeding lines for background selection. Additionally, high-resolution microarrays will be similarly informative to re-sequencing approaches when selected SNPs cover enough genetic diversity of studied genetic materials. Once rare alleles in some genomic regions or germplasm accessions have been identified by broader sequencing of diversity panels, an SNP array can be expanded to cover most SNPs of interest [58].

2.3 Genotyping Using Next-Generation Sequencing Technologies

Recent development of next-generation sequencing (NGS) technologies has provided another efficient and inexpensive class of genotyping tools for whole-genome examination. The high-throughput capacity and significantly reduced cost of NGS technologies, such as 454, HiSeq, SoLiD, have made whole-genome sequencing feasible in regular laboratories, especially for re-sequencing purposes in those species that beneficiate from a complete genome sequence in public databases, such as rice, corn, sorghum, and tomato. With rapid advances in NGS technologies, the traditional two-step paradigm of SNP discovery and subsequent assay is now merging into a single simultaneous process [35, 59–62], in which bioinformatic tools were developed to analyze the sequence data for both SNP discovery and genotyping. The biggest advantage of re-sequencing target regions or genes in genotyping is that the sequence information has an enormous power of differentiating large number of alleles. The resolution of allele differentiation between genotypes depends on sequencing coverage in NGS; the deeper does the sequencing cover, the more detailed is the analysis. Genotyping via NGS has no limit in the power of differentiation between any selected lines as long as deep sequencing is allowed. On the contrary, the number of SNPs is fixed on microarrays and it has to be big enough to present a good set of polymorphic markers for an even coverage of the whole genome of the interested genotypes. The proportion of polymorphic markers in some germplasm lines may be very low if SNPs on a microarray do not represent the genetic diversities of the studied materials. For example, a fixed array designed for genotyping *indica* lines may have poor performance in *japonica* lines. Also, rare alleles usually are not placed on a chip and with NGS they are simply detected during the analysis.

The following limitations of genotyping via NGS need to be overcome before it becomes economically and logistically feasible for rice breeders to practice [63]: (1) Because re-sequencing technology generally depends on alignment of short reads to a reference genome, SNPs falling in repetitive regions or in regions not existing in the reference genome will be hardly detected and analyzed. Novel genes, gene duplications, transposable elements, chromosomal rearrangements, or other stretch of DNA structural variants that occurs in an interested accession as compared to reference genome will fall into this category [64]. This problem may be solved by new re-sequencing technologies which expand the size of sequenced libraries and allow for de novo assembly of short sequence reads derived from variable length clones (such as bacterial artificial chromosomes, BACs). In rice, the *Oryza* Alignment Project (OMAP) is constructing deep-coverage large-insert BAC libraries from 12 *Oryza* species, end-sequencing the clones, and constructing physical maps of all 12 genomes [65–67], which will enable researchers to construct new reference genomes by combining re-sequencing technology and a BAC-pool approach. (2) Although the cost of NGS approach

is rapidly coming down, the huge amount of data generated requires increasingly sophisticated bioinformatic tools [68–70]. The deeper genome (re-)sequencing goes, the more complicated the data analysis are. When thousands of rice accessions need to be surveyed, lower genome sequencing coverage would be an option. In this case, relatively low-quality data and sequencing errors must be carefully screened and discarded to minimize their misleading effect [59]. Low sequencing coverage has many gaps in the scanned genome, and the missing alleles in those blank regions can be predicted using bioinformatic imputations. Therefore, novel algorithms need be developed for genotyping via NGS in rice, and combined utilization of SNP arrays for target regions with high-throughput genome sequencing may be a good approach for genomics-based breeding. Examples of new strategies developed for genotyping via NGS are highlighted below.

The first successful application of deep sequencing to genotyping was in *Arabidopsis* to analyze an F_3 mutant line and its parent Kro-0 [71]. Twenty-five-fold sequence coverage was obtained and 4,023 SNPs with high confidence in an F_3 pool were detected against the reference Col-0. 531 SNPs potentially changed coding sequences, and only one showed polymorphism between the mutant and Kro-0 parental line [71]. Deep sequencing offers high sensitivity and specificity of genetic variation detections but is also expensive. SHOREmap was another scheme including a bioinformatic software kit that supported genotyping and candidate gene identification through mining deep sequencing data of a large pool of recombinants [72]. Recently, a similar strategy is developed, named MutMap, using NGS to identify SNPs responsible for mutant phenotype in rice [73].

In addition to sequence the bulked DNA pools of a segregating F_2 population, low-coverage sequencing of recombinant inbred lines was also performed in rice [61, 74]. In the cross between *indica* cv. 9311 and *japonica* cv. Nipponbare, 150 RILs were selected and sequenced for ~0.02-fold coverage each line, and genotypes at a resolution of about 40 kb for each line were imputed based on recombination and whole-genome sequences of two parents. With the genotyping results, they identified a QTL controlling plant height [61]. In another cross between two *indica* subspecies, Zhenshan 97 and Minghui 63, 238 RILs were sequenced for ~0.055-fold coverage each line. Given that no parents were sequenced, genotypes of parents were first inferred using maximum parsimony of recombination and then genotypes of each line were imputed using a hidden Markov model at a resolution of about 200 kb. QTL mapping was performed and *GW5* gene controlling grain width was identified [74]. An ultrahigh-density genetic map was constructed based on SNPs derived from the low-coverage sequencing data of a rice RIL population following the method used in Xie et al. [74] and was compared with the previous genetic map constructed for the same population using the traditional RFLP/SSR markers [75]. The SNP map was very accurate and its effectiveness for gene mapping and QTL identification was high. Its power of genetic variation detection and resolution for QTL mapping of complex traits such as yield-related traits was demonstrated advantageous as compared to RFLP/SSR maps [75].

Re-sequencing with medium-density genome coverage was performed on 517 rice landraces and a data-imputation method was applied on the 3.6 M SNPs. Thus a high-density haplotype map was constructed and genome-wide association mapping was performed for 14 traits [35]. Then, this methodology was extended to a larger and more diverse sample of 950 worldwide rice varieties and a total of 32 new loci associated with flowering time and with ten grain-related traits [62]. These studies showed the power of NGS as a tool for dissecting complex traits in rice and a good platform for genotyping breeding materials.

Another strategy involves restriction enzyme-mediated digestion of interested genomes to reduce the complexity of the targets and subsequent sequencing the representative libraries (RRL) [76] that is alternatively called RAD tags [77]. RRL or RAD-tag libraries are constructed by digesting genomic DNA with a particular

enzyme or enzyme combination, and the complexity of the genome is reduced. Sequencing the complexity-reduced genome significantly lowers the cost of NGS and effectively captures the informative SNPs. This strategy is particularly valuable for species with large genomes and repeat-rich regions, such as wheat [78], maize [79], and soybean [80], for non-model organisms [81] and for ecological population genomics [82].

RNA sequencing (RNA-Seq) analysis of next-generation sequence data offers an excellent opportunity for SNP discovery. This strategy has been successfully applied in maize by using the 454 system to sequence the transcriptome of shoot meristems [83], in soybean, using Illumina NGS to sequence the RNA samples of shoots and leaves [84], and in oilseed rape, using Solexa to sequence the RNA samples of leaves from seedlings at the six-leaf stage [85]. However, the RNA composition depends on gene expression levels in the harvested tissues and varies at different development stages.

2.4 Other Molecular Marker Platforms

In addition to the molecular marker assay platforms discussed above, a few other technologies were also developed for genotyping breeding materials. Ebana et al. designed 1,578 primer pairs from predicted gene sequences of rice cv. Nipponbare that are positioned roughly one pair every 100–300 kb in the genome (RAPDB, http://rapdb.dna.affrc.go.jp/) to generate ~567 bp amplicons in 140 accessions of rice germplasm and subsequently sequenced the PCR products using Sanger sequencer [86]. They identified 4,357 SNPs, about 3 SNPs per sequenced site on average, or 4.87 SNPs/kb across the genome. This sequence dataset was used for population structure analysis and to evaluate the diversity of different subpopulations of *O. sativa*. Information about the SNPs and their neighboring sequences can be found at the National Institute of Agrobiological Sciences Oryza SNP Database 5 (http://oryza-snp.dna.affrc.go.jp/en/index_en.html).

Masouleh et al. [87] used a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry system (Sequenom, MassARRAY, San Diego, CA, USA) to analyze homozygous and heterozygous samples of rice [87]. They used a multiplex of eight SNPs to tag two agronomic and three grain-quality traits and detected allele variations in 25 rice cultivars. In the study, the correlation between the results of uniplex and eight-plex assays was 98 %, and missed calls were 0.15 % and 1.68 % in uniplex and eight-plex assays, respectively. They suggested multiplex of up to 3,072 SNP assays to be performed in 384-well plate and used as a tool for MAS, which has the ability to detect alleles accurately in hybrids, heterozygotes, and polyploids.

An optical thin-film biosensor chip platform was developed to detect unique transgenes in genetically modified crops or SNP markers in rice [88]. Biotinylated PCR amplicons of the target DNA sequences were hybridized to aldehyde-attached oligonucleotide probes that were anchored on the chip surface of a hydrazine-derivatized biosensor. In the assay, sequences are hybridized to multiple biotinylated detector probes and a thermostable DNA ligase that can detect a single-nucleotide mismatch and recognized by different colors on the chip surface. The assay can be completed within 30 min with highly sensitive and accurate identification of PCR targets, which have advantage of flexibility from low to high throughput and very economical, thus have a potential to be widely used in crop breeding and trait mappings.

High-resolution melting (HRM) is a new method for mutation scanning and genotyping [89]. HRM uses the special fluorescent dyes that can bind to double-stranded DNA and is able to detect variation of PCR amplicons as small as one base difference between samples. The technology has advantages of simplicity, high throughput, high efficiency, and labor-saving. This technology has been widely implemented in human clinical study [90–94]. Recently, HRM has been tested in crop plants, including barley [95, 96], wheat [97], maize [98, 99], rice [100],

and other species [101–103]. HRM is an efficient method complementary with capillary electrophoresis and has a potential to be used in crop breeding.

3 Genomics-Based Breeding Strategies

With the dramatic increase of rice whole-genome sequence data, rapid development of different throughput marker detection platforms, and rich knowledge gained in functional genes, genomics-based breeding is becoming a sound practice. Ideally, we can design genotypes at the whole-genome level and achieve our breeding goal via a combined utilization of various throughput marker platforms with an optimized efficiency. However, we are still far away from understanding the function of all the genes and their interactions involved in trait development, and their performances influenced by the environment. So, the success of strategies for genomics-based breeding will reside in the joint effort of genome-wide marker detection and field phenotyping. Low-, medium- and high-resolution marker assays can be implemented to meet the various needs in rice breeding programs. Taking backcross as an example, breeders can perform high-resolution marker assays to screen for germplasm lines that carry particular alleles of interest and then apply low-resolution marker assays to selection for target genes/QTL and medium- or high-resolution marker assays to background selection of interested individuals in the segregation populations. Thoughtful considerations should be taken in the process of choosing informative markers to increase efficiency and reduce costs. Selection of informative markers used in genotyping of various breeding materials for different purposes is a key step in genomics-based breeding.

3.1 Marker-Assisted Selection for Trait Integration

MAS may greatly increase the efficiency and effectiveness of rice breeding compared to conventional practices. By using molecular markers linked to target genomic regions, desired individuals may be identified based on genotype screen rather than phenotype selection (especially for those traits whose performances largely rely on growth conditions). Therefore, the advantages of MAS include [104]:

- Time saving (substitution of complex field trials, selection of genotypes at seedling stage, high-throughput selection, and faster recovery of the recurrent genome)
- Cost saving (especially for traits that are expensive to score but not only)
- Breakdown of linkage drag (avoid the transfer of undesirable or deleterious genes)
- Powerful and reliable selection (selecting for traits with low heritability, phenotypic evaluation is not feasible or unreliable)
- Gene or QTL pyramiding (simultaneously integrating multiple genes/QTL for a particular trait or multiple traits)

3.1.1 Applications of Genomics-Based MAS in Breeding

MAS has numerous applications in rice and subsequently enhances breeding efficiency.

- Seed identity check. When seeds of different strains are mixed during large breeding programs, genomic markers can be used to simply and quickly identify varieties. In hybrid rice, markers can be used to determine whether putative hybrids are genuine. Multiple F_1s can be also easily screened and desirable genotypes can be selected.
- Assessment of genetic diversity and parental selection. Broadening the genetic base of core breeding material requires the identification of diverse strains to be crossed with elite cultivars. DNA markers have also been used in rice hybrid development for heterosis prediction. There has been extensive research on genetic diversity investigation and parental line selection using genomic markers.
- Marker-evaluated selection (MES). This approach can be used to identify genomic LD under selection (i.e., allelic shifts) of breeding populations using a modified bulked-population breeding system in target environments [105].
- Marker-assisted backcrossing (MABC). MABC is the process of using markers to

select for target loci, minimize the length of the donor segment containing a target locus, and/or accelerate the recovery of the recurrent parent genome during backcrossing [106]. The three steps of selection have been referred to as foreground, recombinant, and background selection, respectively [107]. In these three steps, use of genomic markers greatly improves the efficiency of selection for target genes and genetic background.

- Trait pyramiding. Conventional breeding methods can hardly perform simultaneous selections for multiple traits, such as high yield, good quality, and stress tolerance, because they need different environmental conditions for evaluation. Nevertheless, in the absence of appropriate conditions, multiple disease resistance genes were stacked using MAS strategy [108].
- Screening for transgenes. MAS is a critic tool in screens for functional transgenes or transgenic contaminations.

3.1.2 Considerations About Trait-Linked Markers for MAS

The development of useful markers tightly linked to target genes/QTL is a prerequisite of MAS. It has been widely recognized that confirmation of QTL mapping and validation of linked markers are necessary after primary QTL mapping and prior to MAS. Markers should be tightly linked to target loci, preferably less than 1 cM genetic distance. The use of flanking markers or intragenic markers will greatly increase the reliability of the prediction of expected phenotypes.

The following steps may be taken in the selection of markers for MAS:

- Fine map traits/QTL (use a large recombinant population and a high-density genetic map to identify tightly linked markers)
- Clone the target gene (identify functional markers inside the gene)
- Validate the linked markers (test marker polymorphisms in the intended breeding populations)
- Marker conversion if necessary (some markers have low reproducibility (such as RAPD) and others need complicated, time-consuming, and expensive assays (AFLP and RFLP), and it is better to convert them into breeder-friendly markers)

3.2 Genome-Wide Selection for an Ideal Genotype

3.2.1 Create an Ideal Genome Haplotype (Breeding by Design)

The development of a green super variety is a process of optimization of many important traits involved in yield, grain quality, bio/abiotic stress tolerance, nutrient/water use efficiency, heat/cold tolerance, etc. [109]. The formation of each trait is the result of coordinated interactions between many related genes although in most cases the genetic variations of the involved genes do not all occur in the studied materials. The complete genome sequence and characterization of functional genes in rice permit the molecular design of an ideal genotype. Furthermore, the development of high-throughput marker assay platforms makes the screen for an ideal genotype feasible.

The concept of "Breeding by Design" was proposed by Peleman and van der Vort [110]; it aims to control all allelic variations for all genes of agronomic importance. There are three major steps involved in breeding by design [111]. The first step is to identify genes/QTLs or chromosome segments affecting breeding traits and to study gene effects and gene interactions. The second step is to design the target genotypes for various breeding objectives in various ecological regions. The third step is to identify the most efficient breeding strategies leading to an ideal genotype. Through these steps, breeders are aimed to create novel varieties that consist of desired genome *haplotypes* (Fig. 22.1).

Through a breeding-by-design approach, Wang et al. employed a population of 65 non-idealized chromosome segment substitution lines (CSSLs) to identify QTL and used marker-QTL associations to improve rice quality [112]. They used a novel mapping method named RSTEP-LRT to identify QTL with addictive effect for two quality traits, the area of chalky endosperm (ACE) and amylose content (AC), characterized in eight different environments. Then, they

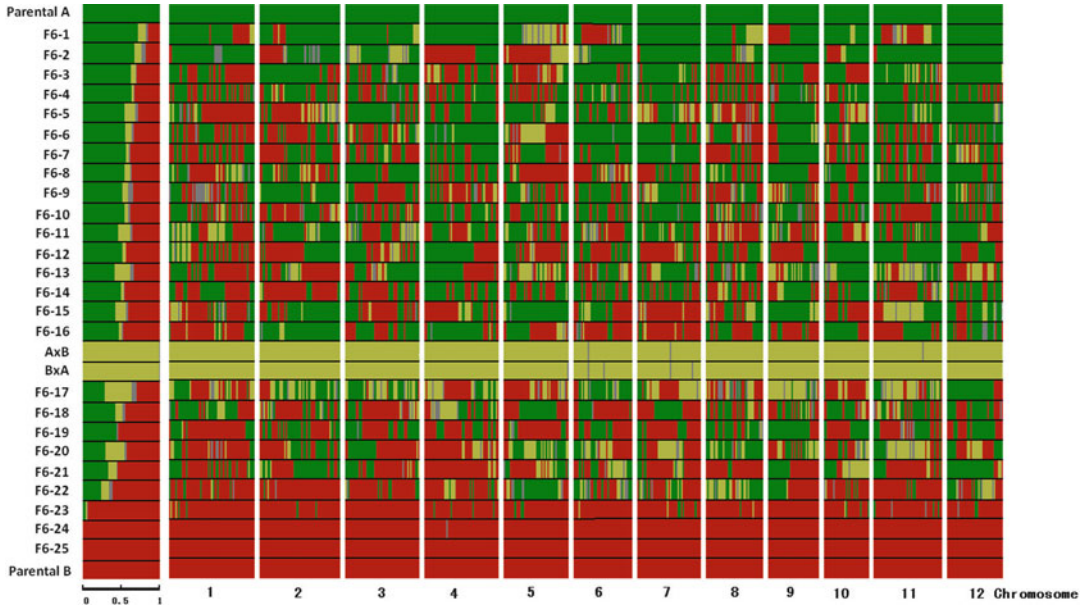

Fig. 22.1 Genome-wide screen for the best recombinants. It shows the screening in a segregating population of rice through graphical genotyping based on high-density marker information. The 12 chromosomes are represented vertically, and the genotypes are represented horizontally. The first column shows the ratio of alleles from each parental line. Parental A and B indicate breeding paternal and maternal varieties. A × B, B × A indicate crossed F_1 plants, and F6-# series indicate different recombinant lines. SNP alleles identical to parental A are labeled as *green* (AA), identical to parental B are labeled as *red* (BB), heterozygotes are labeled as *yellow* (AB), and missing information are labeled as *grey*

designed the target genotype based on the established marker-QTL associations, achieved the target genotype by optimum crossing strategies and developed high grain-quality (low ACE and high AC) lines [112]. In another study, Wang et al. designed a breeding strategy for pyramiding nine target genes to create an ideal genotype in wheat [113]. Wei et al. used genetic information related to the determination of heading date to design genotypes for improving the growth duration and to develop cultivars with target growth duration by quantitative trait locus (QTL) pyramiding [114]. With more QTL identified in rice for combining ability and heterosis of agronomic traits [115] and developing heterosis-prediction models [116], the efficiency of breeding-by-design will be greatly enhanced.

3.2.2 Screen for Genetic Background

It is a highly preferred strategy to integrate a desired trait from an ideal germplasm strain into a popular variety without disturbing the coherence of optimized genetic background for the further improvement of its general performance by removing the limiting factors, such as susceptible to diseases and poor quality. Two processes need to be monitored during trait introgression: (1) to define the introgression region as small as possible, the ideal case being to pinpoint a functional gene underlying the trait and to eliminate all the undesired background around the gene to avoid any adverse genetic drags, and (2) to restore the genetic background of the recipient to its original level (Fig. 22.2). Cleaning the genetic background in the breeding programs for target trait improvement is essential. Genomics-based breeding technologies provide breeders with much needed tools in genetic background selection. Theoretical genetics tell us that the rate of the genetic background of donor parent is expected to decrease by 50 % on average at each generation of backcross. In practice, such a speed is hard to achieve because of genetic segregation of the background genes. Whole-genome SNP chip are

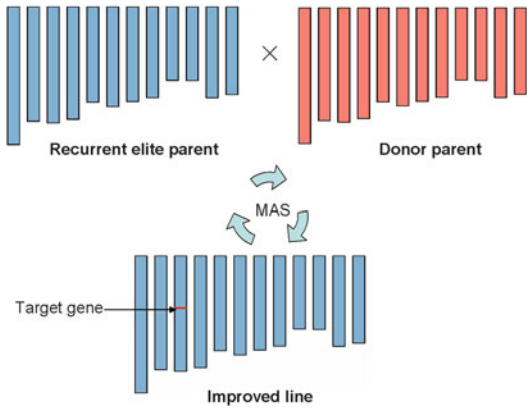

Fig. 22.2 Schematic diagram of target trait improvement. An elite variety is used as recipient parent and crossed with a donor parent that has a desired trait. An improved variety is developed through a few generations of backcrosses with foreground MAS for target trait and genome-wide marker screen for genetic background. Improved variety has a small genomic fragment from donor parent (*red-highlighted*) containing the desired trait but low probability of adverse genetic drags

very useful to screen segregating populations in backcrosses to identify individuals for the best recovery of the ideal genetic background.

In the process of bacterial blight resistance improvement of "Minghui 63," Chen et al. [117] used 128 RFLP markers to recover the genetic background of Minghui 63, and in BC_3F_1, they obtained two plants homozygous for the Minghui 63 genotypes at all marker loci except the RG103 locus residing in the *Xa21* gene region. It meant that the resulting improved version of Minghui 63, or "Minghui 63 (*Xa21*)," was exactly the same as the original except for a fragment of less than 3.8 cM in length surrounding the *Xa21* locus [117]. In the process of introgressing blast-resistant gene *Pi1* to Zhenshan 97, Liu et al. used an SSR marker adjacent to the tightly linked markers of the gene to screen recombinants and to limit the size of donor segment, and then they used ISSR markers to screen the genetic background of the improved Zhenshan 97 [118]. The highest recovery of the Zhenshan 97 genetic background was 97.01 % after the assay of 167 polymorphic bands [119]. used phenotypic and MAS to improve bacterial blight (BB) resistance (*xa13* and *Xa21*) and Basmati quality (*PB-1*) in rice. Background analysis using 252 polymorphic AFLP markers detected 80.4–86.7 % recurrent parent alleles in BC_1F_3 selections, and recombinants having enhanced resistance to BB, Basmati quality, and desirable agronomic traits were identified [119]. However, these marker systems are not sufficiently accurate or efficient for genetic background screen. More high-throughput and high-density marker systems are needed for whole-genome selection.

4 Successful Stories of Genomics-Based Breeding in Rice

Recent advances in rice functional genomics have facilitated genomics-based breeding in rice for stacking of key traits in elite varieties [120]. Bacterial blight (BB), rice blast, brown planthopper (BPH), and stem borer are the most serious diseases/pests in rice and cause heavy losses of rice production worldwide. Compared to the conventional methods of disease/pest controls via chemical sprays, the use of resistant varieties has been recognized as the most economically efficient and environmentally safe ways. More than 32 BB resistance genes, 70 blast resistance genes, 24 BPH resistance genes have been mapped in rice so far [121–124], and a large number of markers that are tightly linked to these genes have been identified or developed, which lay a solid foundation for MAS in rice breeding. To obtain durable and enhanced resistance to BB, three dominant resistance genes, *Xa21*, *Xa7*, and *Xa23*, and two recessive resistance genes, *xa5* and *xa13*, have been frequently introgressed or pyramided into elite rice varieties via genomics-based breeding.

Yuqing He's lab has pyramided *Xa21* and *Xa7* into an elite restorer line, Minghui 63, to improve its resistance to BB [117, 125, 126]. Recently they stacked *Bph14* and *Bph15* in Minghui 63 to enhance its resistance to BPH [127]. Additionally, they successfully tried genomics-based breeding strategies in the improvement of widely used maintainer lines (including Zhenshan 97B, Jin 23B, and Chuanxiang 29B), for better blast and

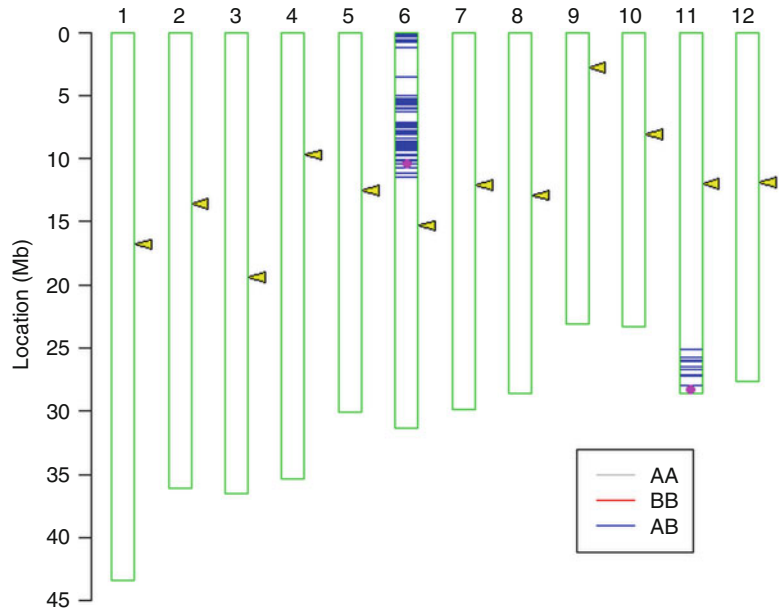

Fig. 22.3 Genetic background screen using RICE6K breeding chip. The 12 chromosomes of rice are labeled from 1 to 12 and the *triangles* indicate the centromeres. The reference genome is Nipponbare MSU v.6.1. The *red dots* indicate the positions of the two target genes, *Pi1* on chromosome 11 and *Pi2* in chromosome 6, respectively. The *blue lines* indicate the positions of the SNPs with heterozygous genotypes where genomic fragments of the donor parent were introgressed and the rest of the genomic regions returned to recurrent parent Kongyu 131

BPH resistance, and grain quality by pyramiding *Pi1*, *Pi2*, *Bph14*, *Bph15*, *Wx*, *ALK*, and *fgr* [128, 129]. These improved maintainer lines were then used as recurrent parent to consecutively backcross to the corresponding male sterile (cytoplasmic male sterility, CMS) lines to obtain improved CMS lines. The improved CMS lines (renamed as Hua2048A, Hua1517A and Hua1971A) have been tested in large-scale field trials to develop better hybrid varieties. The new hybrids with pyramid BPH genes showed significant better resistance to BPH [130].

To optimize genetic background and reduce the adverse genetic drags, a medium-density SNP array, Illumina Infinium RICE6K breeding chip, has been developed and used to screen the backcrossing progenies (Yu et al., unpublished data). Two blast-resistant genes, *Pi1* and *Pi2*, were introgressed from *indica* lines into an elite *japonica* variety Kongyu 131 through MAS of target genes and background screen with RICE6K, as well as phenotype confirmation in the field. In the BC_4F_1, 29 lines were selected and then tested using RICE6K chip. The result showed that the genetic background of the majority of the materials were almost identical to the recurrent parent Kongyu 131, except for the two genomic regions containing *Pi1* and *Pi2*, respectively. The chip screen for cleaner genetic background showed that adverse genetic drags were still linked to the target genes although the rest of the background was quite clean already in BC_4F_1 (Fig. 22.3). Further recombinant selection needs to be performed to break the target genes from the donor genomic fragments.

5 Perspectives

The implementation of genomics-based breeding technologies in rice cultivar development partly relies on the consolidation of seed business in Asia where many small seed companies operate and can hardly afford to set up their own research and development (R&D) centers with art of facilities for genomics-based breeding. In China,

there are over 6,000 small seed companies by 2013 and their revenues are mainly from business of rice and corn hybrid seeds. Majority of those companies do not have their own R&D centers yet. They depend on contracted individual breeding groups to develop new cultivars. Individual breeding groups have little capabilities in genomic research, neither. Therefore, it is of prime importance to build centralized genomics technology centers with the specific goal of serving plant breeders for the implementation of genomics-based breeding technologies in cultivar development.

Genomics-based technologies can be used in each process of a commercial breeding pipeline. Genome re-sequencing- or SNP array-aided germplasm characterization will greatly empower plant breeders to identify genes, QTL, or LD controlling target traits and thus optimize the sufficient utilization of germplasm resources. High-density marker-assisted genetic fingerprinting of elite parental line collections will help validate heterotic group classifications and facilitate heterosis prediction. The practice of accurately predicting quantitative trait phenotypes from information of high-density marker-assisted genotyping will greatly enhance plant breeding efficiency. The advent of SNP array and re-sequencing platforms for rice has enabled genotyping large numbers of individuals using dense panels of markers spanning the genome. Genome-based prediction uses entire genomic variability captured by the available marker set to explain phenotypic variation, instead of relying on selection of single locus based on significance tests, and thus make heterosis prediction feasible and more reliable. This strategy results in a new method other than MAS which named genomic selection (GS) [131]. GS uses a "training population" of individuals that have been both genotyped and phenotyped to develop a model, and produces genomic estimated breeding values (GEBVs) based on genotypic data from a "candidate population" of untested individuals. GS has many advantages compared to MAS: (1) GS estimates all marker effects simultaneously, thus has better accuracy than QTL or GWAS that only focus on "significant" loci with artificial selections and (2) GS builds a model from training populations and then applies the same model on the genotyped test individuals; thus marker effects will be applied directly on target individuals, when QTL results vary greatly between populations and weaken its practicability. Consequence of GS strategy, a main challenge is to estimate all marker predictor effects, p, with available observations, n, when $p > n$. To overcome this problem, a variety of methods, e.g., best linear unbiased prediction [132], ridge regression [133], Bayesian regression [131], kernel regression [134], and machine learning methods [135], have been proposed to develop prediction models for GS. Including these methods, BayesB and GBLUP models using high-density SNP arrays have presented successful examples for this type of approach [136–139].

Target gene introgression through backcrossing is frequently used by plant breeders for single trait improvement. Using high-throughput MAS in large backcross populations can help breeders quickly introduce the target gene into the recurrent parent with little adverse genetic drag. The genetic background can also be cleaned with the aid of whole-genome SNP array to make sure that the overall performance of the improved cultivar remains identical to that of the original parent's. Genomics-based technologies will significantly reduce number of backcrossing cycles needed for target trait improvement.

The knowledge in genomics and gene-trait associations has been accumulating exponentially and various softwares or databases have been developed to exploit the large dataset either available in genome browsers or obtained directly from high-throughput genomic facilities (NAR database summary, available at http://www.oxfordjournals.org/nar/database/c). The design of ideal genotypes with the aid of bioinformatic tools will become a common practice in plant breeding programs. The blueprint will be enforced through the identification and selection of target genes/QTL by using genomics-based technologies. Genetic fingerprinting of commercial seeds using molecular markers will also greatly improve the quality control processes in both efficiency and accuracy.

The implementation of genomics-based technology in rice breeding will not only change breeding practice but also accelerate the formation of large seed corporations in Asia. High-throughput molecular marker platforms will progressively play a major role in the rice breeding programs of large seed companies and also help individual rice breeding groups to improve their efficiency in the selection of ideal genotypes.

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Utilization of Exotic Germplasm

23

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Extensive efforts of the past decades in germplasm collection and conservation have resulted in ~225,000 rice germplasm accessions maintained in the worldwide rice genebanks today, which include ~66 % landraces and ~24 % modern varieties of two major cultivated species, *O. sativa* and *O. glaberrima*, plus ~10 % accessions of 22 wild species [3]. While this rich source of rice germplasm is holding almost all genetic diversity for rice improvement, only a very small portion (<5 %) of the huge numbers of germplasm accessions has been utilized in rice improvement [16] because most rice germplasm accessions are considered exotic. To rice breeders, exotic rice germplasm accessions may consist of four major types: (1) wild rice species, (2) different subspecies (*indica* vs. *japonica*), (3) traditional landraces, and (4) unadapted modern varieties of the same subspecies. According to Duvick [14], breeders are reluctant to utilize exotic germplasm largely because of three main reasons. First, slow but consistent progress can be achieved even within a narrow genetic basis of many breeding populations. Second, outstanding favorable phenotypes of commercial varieties tend to be destroyed in crosses involving unadapted exotic parents, particularly parents of different species and subspecies are used. A third reason is that selection of exotic germplasm as parents of breeding programs by breeders has been largely based on phenotype. In practice, only few exotic germplasm accessions with "extreme phenotype" for target traits tend to be chosen as parents of breeding populations. The underutilization of the germplasm resources in breeding programs may have serious consequences, including reduced genetic variation, particularly novel genetic variation in breeding populations, slow responses to selection, and the narrow genetic basis and thus vulnerability to biotic and abiotic stresses of developed cultivars. For example, while intensive artificial selection in modern plant breeding since the "Green Revolution" (GR) in 1960s has dramatically increased the productivity of rice under the modern high-input agriculture conditions [33], the yield potentials of modern inbred and hybrid rice cultivars have apparently reached respective plateaus over 20 years [6, 64]. Also, the realized yields of modern semidwarf rice cultivars are far from their potentials in farmers' fields due to many abiotic and biotic stresses. In particular, approximately 60 % of rice grown in the rainfed areas of Asia remains landraces because most modern high yield semidwarf varieties are not adapted to the unfavorable rainfed environments (S. Pandey, personal communication). To break yield ceiling and

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improve yield stability, genetic base broadening [72] or gene pool enrichment [17] has been proposed to transfer useful genes from unadapted exotic germplasm into elite backgrounds in crop improvement. Unfortunately, this strategy has never been seriously practiced in rice. In this chapter, we will focus on the current status in utilizing exotic germplasm for rice improvement and major challenges in this area that can ultimately be resolved by the progresses in rice genomic research described in previous chapters.

1 The Values of Exotic Germplasm in Breeding

Although the above grouping of exotic rice germplasm into four major types is somewhat arbitrary and it is difficult to determine their full genetic potential in rice improvement, empirical knowledge from past breeding practices has revealed unique values of each type in rice improvement.

Wild rice species: Within the genus of *Oryza*, there are 24 different species (see Chap. 2). These include two cultivated diploid species with the AA genome, *O. sativa* and *O. glaberrima*; eight AA genome diploid wild species belonging to the *O. sativa* complex and six diploid wild species of different genomes (BB, CC, EE, FF, and GG) belonging to *O. officinalis* or *O. meyeriana* complex; plus seven tetraploid species of different genomic composition. Historically, these wild rice species have been a valuable source for many important traits in rice improvement (Table 23.1). These traits include resistances to different biotic stresses such as diseases (blast [2, 12, 32], bacterial leaf blight (BLB) [18, 30, 70, 74, 80, 95], viruses [35, 36, 61, 77], and brown planthopper (BPH) [13, 22–26, 28, 31, 38, 48, 53, 66–68, 87], nematode [49], whiteback planthopper [75], etc.), abiotic stresses, e.g. aluminum toxicity tolerance [62], as well as cytoplasmic male sterility [47] and yield components [20, 52, 59, 79, 83]. Genes controlling these highly heritable traits, once introgressed from wild species into *O. sativa*, can be easily identified, mapped, and even cloned using DNA markers (see Chaps. 11 and 12). Interestingly, genes conferring resistances to blast, BLB, and BPH from wild species appear to have broader spectrums of resistances to most races or strains of the pests. Well-known examples include *Xa21* from *O. longistaminata* [27, 69], *Xa23* from *O. rufipogon* [93], *Pi9* from *O. minuta* [2], and *Pi54rh* from *O. rhizomatis* [12]. Cloning of these resistance genes has tremendously facilitated their uses in rice improvement with MAS (Chap. 23) or transgenic approach (Chap. 25).

Direct uses of wild species for improving complex traits such as abiotic stress tolerances and yield/quality traits of *O. sativa* have been very limited in most rice breeding programs, largely because of the difficulty in overcoming severe pre- and post-reproductive barriers of interspecific crosses involving parents of different genomes that block the gene flow from most wild species accessions into *O. sativa* [63]. However, several recent studies have identified QTL in *O. rufipogon* accessions that can enhance yield performances of some *O. sativa* varieties [4, 20, 52, 54, 71, 78, 84]. While these results clearly indicate the presence of useful genetic diversity in the wild rice species for enhancing rice yield, the real values of these yield-enhancing QTLs from some accessions of *O. rufipogon* have yet to be confirmed in more high yielding genetic backgrounds of *O. sativa*. Also, it remains unknown if these yield-enhancing alleles from wild rice species are unique and absent in the cultivated gene pools of rice.

Different subspecific accessions: The *indica-japonica* subspecific differentiation within *O. sativa* has long been recognized with *indicas* distributed primarily in the tropical and subtropical areas and *japonicas* mainly in the temperate zones and high elevation areas of hilly mountains [7, 63]. This subspecific differentiation within *O. sativa* has been well characterized at both phenotypic and molecular levels [8, 41, 46, 94]. Most *indica-japonica* F_1 hybrids exhibit high levels of heterosis in their vegetative growth, but not in their yield performances because of varied degrees of hybrid sterility [40, 63]. Many earlier efforts used mapping populations derived from inter-subspecific crosses to genetically dissect

Table 23.1 Summarized results of valuable traits and genes/QTL identified through introgression from exotic germplasm into cultivated rice (*O. sativa* L.)

Trait	Gene/QTL	Chr	Donor species	References
Bacterial blight resistance	*Xa-21*	11	*O. longistaminata*	[27]
	Xa-23	11	*O. rufipogon*	[93]
	Xa27	6	*O. minuta*	[2]
	Xa-29(*t*)	1	*O. officinalis*	[74]
	Xa30(*t*)	11	*O. rufipogon*	[80]
	xa32(*t*)	12	*O. granulata*	[70]
	xa-32(*t*)	11	*O. australiensis*	[95]
	Xa35(*t*)	11	*O. minuta*	[18]
Blast resistance	*Pi40*	6	*O. australiensis*	[32]
	Pi9	6	*O. minuta*	[2]
	Pi54rh	11	*O. rhizomatis*	[12]
Sheath blight resistance	N.A.	N.A.	*O. minuta*	[5]
Brown planthopper resistance	*Bph10*	12	*O. australiensis*	[28]
	Bph13	2	*O. eichingeri*	[48]
	Bph12(*t*)	4	*O. latifolia*	[87]
	Bph-13(*t*)	3	*O. officinalis*	[68]
			O. nivara	[53]
	Bph14	3	*O. officinalis*	[26]
	Bph15	4	*O. officinalis*	[26]
	bph-16	4	*O. officinalis*	[23]
	bph-11	3	*O. officinalis*	[22]
	bph-12	4	*O. officinalis*	[22]
	Bph-18	11	*O. australiensis*	[31]
	bph-18(*t*)	4	*O. rufipogon*	[38]
	bph-19(*t*)	12	*O. rufipogon*	[38]
	Bph20(*t*)	4	*O. minuta*	[66]
	Bph21(*t*)	12	*O. minuta*	[66]
	Bph22(*t*)	6	*O. minuta*	[19]
	Bph22(*t*)	N.A.	*O. glaberrima*	[67]
	Bph23(*t*)	N.A.	*O. minuta*	[67]
	QTL(*bph-22*(*t*), *bph-23*(*t*))	4, 8	*O. rufipogon*	[24]
	bph24(*t*)	N.A.	*O. rufipogon*	[13]
	Bph27	4	*O. rufipogon*	[25]
White back planthopper resistance	*Wbph8*	4	*O. officinalis*	[75]
	Wbph7	3	*O. officinalis*	[75]
Nematode resistance	*Hsa-10g*	N.A.	*O. glaberrima*	[49]
Gray stunt resistance	*Gs*	N.A.	*O. nivara*	[35]
Yellow mottle virus resistance	*Rymv1*	4	*O. glaberrima*	[61]
	Rymv2	N.A.	*O. glaberrima*	[77]
Tungro resistance	*RTSV*	N.A.	*O. rufipogon*	[36]
			O. officinalis	
			O. ridleyi	
Yellow stem borer resistance	N.A.	N.A.	*O. longistaminata*	[76]
			O. rufipogon	
Aluminum toxicity tolerance	*QTL*	1, 3, 9	*O. rufipogon*	[62]

(continued)

Table 23.1 (continued)

Trait	Gene/QTL	Chr	Donor species	References
Tolerance to acidity, iron, and aluminum toxicity	N.A.	N.A.	*O. glaberrima*	[34]
Increased stem elongationability	*dw3*	N.A.	*O. rufipogon*	[15]
Weed competitiveness	N.A.	N.A.	*O. glaberrima*	[34]
Drought tolerance	N.A.	N.A.	*O. glaberrima*	[34]
Drought tolerance	*QTL*	N.A.	*O. rufipogon*	[97]
Cytoplasmic male sterility	*cms-WA*	N.A.	*O. sativa* f. *spontanea*	[47]
	cms-IR66707A	N.A.	*O. perennis*	[11]
	orfH79, *cms-HL*	Mitochondrion	*O. rufipogon*	[65]
Yield and its components	*yld1*, *yld2*	N.A.	*O. rufipogon*	[84]
	QTL	N.A.	*O. rufipogon*	[20, 52, 79]
	QTL	N.A.	*O. rufipogon*	[59, 71, 78]
Early growth duration	*QTL*	N.A.	*O. latifolia*	[60]

complex traits ([43]; Chap. 1). The amounts of variation at genomic, genetic, and phenotypic levels revealed in these biparental subspecific populations were remarkably high, resulting in identification of large numbers of genes/QTLs affecting a wide range of complex traits [39, 40, 44–46, 50, 51, 55, 81, 85, 86, 89]. Unfortunately, few successful cases have been reported in applying the QTL identified in the inter-subspecific populations to improving complex traits of rice. One of the major reasons is the fact that expression of most QTL identified in inter-subspecific populations tends to be greatly affected by genetic background [9, 29, 56, 88, 96].

Historically, considerable efforts have been taken to exploit the inter-subspecific variation in rice improvement, but few have been documented. Empirically, it is difficult to identify true superior intermediate types that combine unique desirable traits of both subspecies in segregating populations of *indica-japonica* crosses. Nevertheless, there have been successful cases. One well-known example was the development of the high yielding Tongi type varieties from *indica-japonica* crosses using the backcross (BC) breeding approach by Korean breeders during 1970–1980s. However, these high yielding varieties of Tongi type belong actually to the *indica* type [10]. Another example is that most high quality long-grain cultivars adapted well to south US are *japonicas*, but with introgressed traits from *indica* [41]. In practice, it is difficult to exploit this *indica-japonica* genetic variation using the conventional pedigree breeding approach largely because of the varied degrees of the post-reproductive barriers and the prolonged segregation in the progenies derived from typical *indica-japonica* crosses, resulting apparently from possible cryptic genomic variation and the coadapted *indica* or *japonica* complexes [40, 63]. Thus, this type of activities accounted for a very small portion of the total breeding efforts in any specific breeding program in the past and it remains largely unknown that how much of this huge inter-subspecific variation can be used in rice improvement, particularly for complex traits such as yield and tolerances to abiotic stresses.

Answer to this question came recently from the massive BC breeding efforts in China and IRRI in which introgression of useful genetic diversity from 203 germplasm accessions of 34 countries into elite rice genetic backgrounds has been carried out for improving a wide range of complex traits. These traits include tolerances to drought, salinity, low and high temperatures, submergence, zinc deficiency, and anaerobic germination, and resistances to blast, BLB, sheath blight, BPH, etc. [1, 21, 37, 57, 58]. This introgression breeding strategy has two unique features: (1) the donors of the BC breeding program

Table 23.2 Summary results of the BC breeding program for improving tolerances/resistances to six abiotic and biotic stresses in five elite genetic backgrounds (IR64 and Teqing are *indica*; NPT, Chaoyou 1, and Jigeng88 are *japonica*)

Traits screened	Salinity				Cold (flowering)	Cold (seedling)	Zinc deficiency			Anaerobic germination		
Recipient	IR64	Teqing	NPT	Jigeng88	Chaoyou 1	C418	IR64	Teqing	NPT	IR64	Teqing	NPT
N	58	57	52	4	11	7	48	42	35	44	47	35
Number of *indica* donors	47	47	42	3	8	6	40	33	25	37	38	29
Selected lines	369	345	289	204	73	171	370	320	237	31	67	77
Selection intensity (%)	4.39	3.67	3.44	17.00	3.04	9.50	7.77	8.08	7.90	0.43	0.90	3.41
Number of *japonica* donors	9	9	7	1	3	1	7	6	7	6	8	5
Selected lines	70	66	44	99	51	6	63	63	84	11	14	31
Selection intensity (%)	3.89	3.67	3.14	24.8	4.25	3	7.5	8.75	7.86	0.91	1.03	2.78
Traits screened	Drought				Heat	Drought	Brown planthopper			Submergence		
Recipient	IR64	Teqing	NPT	C418	Chaoyou 1	Jigeng88	IR64	Teqing	NPT	IR64	Teqing	NPT
N	149	100	115	5	11	4	60	64	59	56	57	59
Number of *indica* donors	120	65	64	5	8	3	49	60	49	47	50	50
Selected lines	2,634	613	831	113	238	97	565	221	2	538	431	451
Selection intensity (%)	10.85	3.64	4.24	0.80	6.61	8.10	11.53	3.68	0.04	1.15	0.86	0.90
Number of *japonica* donors	28	16	10	–	3	1	9	11	7	8	7	7
Selected lines	467	58	66	–	86	38	54	21	0	83	52	63
Selection intensity (%)	9.78	3.16	3.25	–	6.37	9.5	6	1.91	0	1	0.74	0.9

N is the total number of BC_2F_2 populations screened (see more details from [1, 37]). In all cases, the recurrent parents were killed by the stresses, and the selection intensity was actually the survival rate (in %) of the BC_2F_2 plants

The levels of stresses in screening the BC_2F_2 populations were very severe and killed the recurrent parents (IR64, Teqing, NPT, C418, Chaoyou 1, and Jigeng88). Thus, the selected survival BC progenies were all transgressive segregants

Table 23.3 Phenotypic values of the percentage of diseased hills (PDH), percentage of diseased panicles (PDP), and percentage of diseased spikelets (PDS) of nine resistant introgression lines (ILs) derived from the cross between Teqing (the *indica* recipient) and Lemont (the *japonica* donor) evaluated under the disease hotspots of false smut (*Ustilaginoidea* virens) in Beijing and Shenyang [98]

	Beijing			Shengyang		
ILs #	PDH (%)	PDP (%)	PDS (%)	PDH (%)	PDP (%)	PDS (%)
121	33.3	17.6	0.4	105	14.3	1.7
103	33.3	14.5	0.3	103	14.3	1.7
191	25	8.3	0	102	14.3	2
40	25	30.2	0.4	103	0	0
114	61.1	16.8	0.3	103	37.5	6.3
123	16.6	7.1	0.4	104	0	0
85	50	26.7	0.2	103	42.9	5.6
2	33.3	41.9	0.4	102	14.3	1.9
188	33.3	14.6	0.4	103	0	0
Teqing	99.6*	72.4**	4.92**	98.5**	21.7**	1.97**
Lemont	33.2	30.2	1.96	16.7	5.8	0.71

*, ** Represent significant differences between the parents at $P < 0.01$ and 0.001, respectively

are equivalent to a mini-core collection of the *O. sativa* gene pool, representing a significant portion of the genetic and geographic diversity within *O. sativa* [90]; and (2) all BC populations were screened for these target traits regardless of the performances of the donors for the target traits. Two general results regarding the useful subspecific genetic variation were obtained and are summarized below.

The most important result is that there are tremendous amounts of genetic diversity between *indica* and *japonica* accessions for all traits investigated. Table 23.2 summarizes the BC breeding results from 1,179 trait/population combinations. Transgressive segregants were identified in most populations, indicating that most donors have genes for the survival of the BC progenies under different stresses [1]. These included 301 trait/population combinations in the *japonica* backgrounds (a New Plant Type line, Chaoyou 1, and Jigeng88) with a diverse set of *indica* donors and 134 trait/population combinations in the *indica* backgrounds (IR64 and Teqing) with a diverse set of *japonica* donors. On average, for any specific traits and recipients, no difference was found between the *indica* and *japonica* donors regarding the frequency of transgressive segregants (selection intensity) in the BC progeny, even though considerable differences in selection intensity existed among specific donors (data not shown) and among different target traits. This result indicates that selection of recipients and appropriate levels of stresses are critically important in improving abiotic stress tolerances using BC breeding. Clearly, there is a rich source of genetic variation in the *indica* gene pool that can be used for improving complex traits of *japonica*, and vice versa. The subspecific differentiation of *indica* and *japonica* within *O. sativa* does not seem to have specific implications regarding the useful genetic variation for any specific traits. Interestingly, complementarity between *indica* and *japonica* appears to be present in race-specific resistance to rice blast. For example, Teqing, an elite *indica* variety developed in Guangdong of China, is highly susceptible to most blast races of south China but is highly resistant to almost all blast races in south US. In contrast, Lemont, a *japonica* variety from south US, is susceptible to most local blast races [73] but highly resistant to most races in south China and thus is used as a resistance donor in *indica* breeding programs (S.C. Zhou, personal communication). It can also be used for improving resistance of *indica* to false smut (Table 23.3), a fungal disease of increasing importance, to which there is no source of high

level resistance in the existing germplasm accessions.

Secondly, this rich subspecific variation is often "hidden" in a sense that most donors can contribute to trait enhancing genes/alleles even though they appear to be inferior for the target traits. To better illustrate this, Table 23.4 shows our BC breeding results for improving cold tolerance (CT) at the reproductive stage and for improving heat tolerance at the flowering stage. In this study, BC populations derived from crosses between a superior recipient *japonica* variety, Chaoyou 1, and 11 donors were screened under constant cold-water irrigation, resulting in selection of 324 introgression lines (ILs) with significantly improved CT, confirmed by progeny testing [57]. Of the 11 donors, eight are cold-sensitive *indicas* from tropics and three are *japonicas* which do not have strong CT either. Clearly, transgressive segregants for CT were identified in all BC populations, and the number of CT progeny and their mean CT level of the selected progeny from each population did not correlate with CT performances of the donors. When the same BC progeny from 9 of the 11 populations were screened for heat tolerance at the flowering stage, 124 introgression lines with significantly improved heat tolerance were obtained and confirmed by progeny testing (Table 23.4 [58]). Again, all 9 donors (6 *indicas* and 3 *japonicas*) contributed to heat-tolerant BC progeny, though none of them are known to have good heat tolerance. Bg90-2 (*indica*) and Yuangeng 7 (*japonica*) were two best donors for heat tolerance in the Chaoyou 1 progeny. This was generally true for a wide range of complex traits such as yield, tolerances to drought, salinity, cold, heat, zinc deficiency, anaerobic germination, submergence, and BPH [1]. A curious finding in these studies was the consistent association of significantly increased plant height in the selected DT and CT progenies in the *japonica* genetic backgrounds, which were not the cases in the *indica* genetic backgrounds [21, 57, 58].

Traditional landraces and unadapted modern varieties of the same subspecies: Traditional landraces and unadapted modern varieties represent two largest groups of rice germplasm accessions within either *indica* or *japonica* subspecies. The two general results on the subspecific variation in the previous section are generally true for this group of germplasm accession (Tables 23.2 and 23.4). Obviously, there are tremendous amounts of genetic variation within the subspecific gene pools of *O. sativa*, which can be used for improving almost any complex traits in rice. In particular, this rich source of genetic variation is largely hidden in most landraces and unadapted accessions of the same subspecies of *O. sativa*. Table 23.5 shows the lesion lengths of ten introgression lines (ILs) and their parents caused by 15 tropical races of the BLB pathogen, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). The recipient, Huang-Hua-Zhan (HHZ), is an elite Chinese *indica* variety resistant to only 5 of the 15 *Xoo* races. Two *indica* donors (PSBRc28 and PSBRc66 from Philippines) are resistant to two and six of the *Xoo* races. Fifteen of the HHZ ILs from the BC_1 populations of HHZ/PSBRc28 and HHZ/PSBRc66 are highly resistant to all 14 *Xoo* races. Similar results were obtained for blast resistance in our introgression breeding program (data not shown). Apparently, this type of hidden genetic diversity is true even for resistance to BLB and blast which are generally believed to be controlled by single major R genes.

2 Conclusion and Future Perspective

In conclusion, past breeding efforts have revealed tremendous amounts of useful genetic variation for almost any traits of rice in the gene pools of *Oryza*, primarily in the exotic germplasm accessions within *O. sativa*. BC breeding and appropriate phenotypic selection are effective ways to exploit the useful genetic variation in the exotic germplasm.

Table 23.4 Screening results of 11 BC_2F_4 backcross populations derived from crosses between a *japonica* variety, Chaoyou 1 (the recurrent parent), and 11 donors for cold tolerance at the booting stage [57] and for heat tolerance at the flowering stage [58]

		Selection for cold tolerance at the booting stage				Selection for heat tolerance at the flowering stage					
				Seed set (%)				Seed set (%)		Spikelets per panicle	
Donor[a]	Origin	*N*	SI (%)[b]	Mean[c]	Range	*N*	SI (%)	Mean[c]	Range	Mean[c]	Range
Bg90-2 (*I*)	Sri Lanka	41	9.11	63.3 d	50.3–86.5	25	6.25	41.3 abc	23.0–77.0	171.2 abc	124.0–253.1
X21 (*I*)	Vietnam	29	6.44	64.3 cd	50.6–87.1	9	2.25	38.1 abcd	19.5–50.0	192.5 ab	143.3–275.5
X22 (*I*)	Vietnam	28	6.22	65.6 bcd	50.7–87.3	–	–	–	–	–	–
Q5 (*I*)	Vietnam	31	6.89	71.1 abc	50.9–91.4	6	1.50	41.1 abc	19.9–75.4	204.6 a	160.4–274.2
Chhomrong (*J*)	Nepal	24	5.33	75.6 a	51.4–87.8	17	4.25	37.7 abcd	19.3–65.5	172.9 abc	115.9–258.8
Doddi (*I*)	India	25	5.56	71.0 abc	50.2–90.0	–	–	–	–	–	–
Feng-Ai-Zhan (*I*)	China	44	9.78	74.1 a	52.2–98.5	12	3.00	46.6 a	31.3–70.1	180.7 abc	138.2–238.0
Shennong265 (*J*)	China	21	4.67	69.2 abcd	50.7–93.8	9	2.25	28.9 d	17.0–44.4	171.5 abc	112.0–216.7
Yuangeng7 (*J*)	China	41	9.11	71.1 abc	50.0–90.1	25	6.25	45.5 ab	23.9–65.6	153.8 c	83.2–255.4
OM997 (*I*)	Vietnam	21	4.67	72.1 ab	54.9–89.6	13	3.25	33.0 cd	17.0–48.9	171.9 abc	111.6–230.4
Cs94 (*I*)	Vietnam	19	4.22	64.2 cd	51.4–86.0	8	2.00	33.6 abcd	24.9–48.3	175.5 abc	121.4–280.4
Chaoyou (*J*)	China	324	6.55	24.8 e	19.0–30.0	124	3.44	5.2 e	0.0–7.9	157.4 bc	127.4–178.6

[a]*I* and *J* represent *indica* and *japonica*, respectively

[b]*N* is the number of cold-tolerant or heat-tolerant BC plants selected from each population and SI = selection intensity

[c]Different letters indicate the statistical significance in seed set at $P < 0.05$, based on the Duncan testing of ANOVA

Table 23.5 Lesion lengths of ten Huang-Hua-Zhan (HHZ) introgression lines and their parents caused by 14 tropical races of the bacterial leaf blight pathogen, *Xanthomonas oryzae* pv. *Oryzae*

Introgression lines/**parents**	Tropical races of bacterial leaf blight (*Xanthomonas oryzae* pv. *Oryzae*)													
	P1	**P2**	**P3b**	**P3c**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9a**	**P10**	**P9c**	**P9b**	**P9d**
HHZ (P1)	31.5	23.2	13.2	25.7	10.4	**2.40**	29.6	*5.04*	8.58	28.5	8.40	28.4	17.1	22.6
PSBRC66 (P2)	*3.40*	18.5	16.4	21.4	11.6	**0.90**	13.0	**2.56**	8.80	**4.06**	7.00	12.0	**3.42**	17.3
HHZ 15-1	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**
HHZ 15-2	**0.20**	**0.78**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**
HHZ 15-3	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**
HHZ 15-4	**0.22**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**
HHZ 15-5	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**2.34**	**2.26**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**
PSBRC28 (P3)	**1.70**	17.2	19.1	21.0	10.0	*3.35*	20.9	*4.25*	7.65	23.1	6.40	20.6	21.7	21.2
HHZ 19-1	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**
HHZ 19-2	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**	**0.20**
HHZ 19-3	**0.22**	**0.66**	**0.94**	**0.50**	**0.20**	**0.20**	**0.20**	**0.20**	**0.42**	**0.20**	**0.32**	**0.92**	**0.20**	**0.20**
HHZ 19-4	**0.20**	**1.00**	**0.56**	**0.26**	**0.20**	**0.20**	**0.20**	**0.20**	**0.28**	**0.20**	**0.68**	**0.26**	**0.20**	**0.20**
HHZ 19-5	**0.16**	**3.12**	**2.24**	**0.64**	**0.38**	**0.40**	**0.80**	**0.52**	**0.30**	**0.34**	**0.28**	**0.62**	**0.36**	**0.36**

The number in **bold** means "HR", while the *italic* indicates "R"

Unfortunately, this rich source of naturally occurring diversity is largely hidden at the phenotypic levels and remains largely uncharacterized at the genomic and molecular levels, and thus very much underutilized in the past rice improvement. The increasing occurrences of biotic and abiotic stresses of rice crops around the world in recent years have called for a second green revolution to develop "Green Super Rice (GSR)" that has high and stable yields under less inputs [92]. Thus, the GSR cultivars under development should have many desirable traits such as resistances/tolerances to multiple abiotic and biotic stresses, in addition to high yield potential. Then, the greatest challenge facing today's rice scientists is to systematically characterize and utilize this hidden genetic diversity in the core collection of the rice germplasm in future genetic, genomic, and breeding research in order to have the best breeding strategies in future rice improvement.

Strategically, it is essential that future rice genetic/functional genomic research be fully integrated with breeding. In the previous chapters, it is clear that tremendous progress has been made in rice genetic and functional genomic research in recent decades, with an ultimate goal to improve the accuracy and effectiveness of rice breeding. However, these achievements have not yet changed much of today's rice breeding activities. For example, few of the cloned rice genes/QTLs of large effect are actually used in rice farmers' fields, because these genes/QTLs are part of the complex systems that control complex phenotypes in rice. As a result, single large-effect genes/QTLs alone have a limited power in predicting genotype–phenotype relationships in breeding populations when different parental lines are used and/or target traits are controlled by segregating loci at different levels of complex signaling pathways [91]. Also, past genetic characterization of complex traits using the QTL mapping approach has almost exclusively used random segregating populations, while breeders are dealing with a small number of individuals with specific target traits selected from segregating breeding populations. Genetically, this small group of selected progeny is expected to contain the most important genetic information regarding specific target traits to breeders. Thus, efforts are needed to understand why and how directional phenotypic selection is operating on the genetic variation of complex traits using materials from real breeding populations. In this regard, a new strategy and theoretical genetics models have

been proposed to characterize the genomewide responses to selection using population genetics parameters and to understand the underlying genetic and molecular mechanisms of complex target traits using breeding materials and advanced molecular/-omic tools [42, 82, 91].

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Transformation and Transgenic Breeding

24

Hao Chen and Yongjun Lin

1 Introduction

As one of the most important food crops, rice improvement contributes greatly to food security of the world. In the last century, rice yield potential achieved two significant leaps associated with two important technological breakthroughs: the adoption of semidwarf varieties since the 1960s and producing rice hybrids since the 1970s [209]. However, rice improvement has reached a plateau in the past 2 decades. For example, rice yield in China has only increased 3.8 % within 11 years from 1998 to 2009 (http://geo.irri.org:8189/wrs/). Moreover, rice production in the new century has been confronting enormous challenges: increasingly frequent occurrence of insects, diseases, and droughts cause serious yield losses annually and huge fertilizer investment conflicts with declining natural resources [222]. To address these challenges, an ambitious breeding goal to develop Green Super Rice (GSR) for sustainable rice production has been proposed, aiming at improving the following agronomically important traits: insect and disease resistance, drought tolerance, nutrient-use efficiency, and quality and yield potential [222].

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The emergence and development of genetic engineering approaches provide new opportunities for rice breeding and realizing the goal of GSR. Rice transformation achieved important successes in the late 1980s. Since then, a number of foreign genes with various origins have been introduced into rice to improve different target traits. In this chapter, we introduce the establishment of the rice transformation system and some promising technologies and methods first from the technical viewpoint and then comprehensively review the main progress in transgenic rice research in accordance with the traits relevant to developing GSR. Finally, the prospects of transgenic rice research are discussed.

2 Rice Transformation

Rice transformation achieved important successes in 1988 [185, 223, 224]. Transgenic rice plants were regenerated from transformed rice protoplast via electroporation-mediated [185, 224] or PEG-mediated methods [223]. Rice transformation via particle bombardment method succeeded in 1991 [25]. Rice transformation via *Agrobacterium*-mediated method was first reported by Chan et al. [17]. Later, a highly efficient *Agrobacterium*-mediated transformation system for *japonica* rice was established by Hiei et al. [53] using mature seed-derived callus as the explant, which has subsequently become the most popular method of rice transformation.

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Significant modifications were made by Toki et al. [184] upon the protocol of Hiei et al. [53] to greatly shorten the transformation procedure. Hiei et al.'s protocol made transformation very amenable for *japonica* rice; however, it remains obstinate for many *indica* varieties. Some modified protocols of *Agrobacterium*-mediated transformation for *indica* rice were published [51, 97]. Hiei and Komari [52] provided a new protocol of *Agrobacterium*-mediated transformation adaptable to both *japonica* and *indica* varieties. The only disadvantage of this protocol is requiring immature embryo as the explant, with the collection and isolation of immature embryos very laborious and limited by the growth season.

Overall, the technological platform of rice transformation has been established. However, the development of rice functional genomics research and transgenic breeding require more technical innovations of rice transformation. The following is a brief review of some special transformation technologies with great potential application in transgenic rice research.

2.1 Gene Silencing

Gene silencing technology is valuable for both gene function research and crop improvement. At early stages, antisense strategies had been applied successfully to suppress both foreign transgenes and endogenous genes in plants even if the exact molecular basis of this strategy remained unclear at that time (reviewed in [11, 116]). The discovery of RNA interference (RNAi) provided more powerful tools for gene silencing [36]. Systematic studies showed that double-stranded RNA (dsRNA) with a self-complementary hairpin structure (hpRNA) is much more effective to trigger RNAi and suppress gene expression than either sense or antisense RNAs [26, 170, 200, 201]. Especially, hpRNA with a spacer of an intron sequence, which can be removed after transcription, is most efficient to induce RNAi [26, 170, 201]. However, one drawback of the hpRNA strategy is the "off-target" effect, because a number of diverse small interference RNAs (siRNAs) processed from a long hpRNA in the plant may induce the gene silencing of both the target and unintended genes (off-targets) as long as siRNAs and silenced genes shared perfect or near-perfect sequence complementarity [136].

Highly specific gene silencing technology using artificial microRNA (amiRNA) was recently developed [85, 117, 157, 199]. Unlike the long hpRNA, an amiRNA precursor only generates a definite 21-nucleotide mature amiRNA product [199], and therefore the potential off-targets can be predicted and avoided when designed by software. A detailed protocol for using amiRNA technology in rice was established by Warthmann et al. [199].

Generally, there are two main ways to deliver RNAi vectors into plants: by stable transformation such as *Agrobacterium*-mediated transformation or by virus-induced gene silencing (VIGS). In VIGS, the target genes of the plant host are silenced after they are infected with the recombinant viral genomes containing the fragments of the host target genes as part of the defense mechanism of the plant against virus infection (reviewed in [158]). VIGS has been widely applied as a powerful tool to study plant gene function as a rapid and low-cost gene silencing method in dicotyledons. However, its use in rice and other monocotyledons is very limited [141]. Few studies of VIGS in rice have been reported so far—using two VIGS vectors, brome mosaic virus and rice tungro bacilliform virus (RTBV) [34, 141, 158].

2.2 Gene Targeting

Gene targeting refers to the alteration of a specific DNA sequence in an endogenous gene at its original locus in the genome [68]. Gene targeting is highly desired for gene function validation and transgenic breeding because it does not change the genomic structure. One of the main strategies for gene targeting in higher plants is based on homologous recombination (HR). However, the occurrence incidence of gene targeting based on HR is extremely low (in the order of only 10^{-3} to 10^{-6}) compared to the random integration via nonhomologous end-joining, which is the main

technical obstacle to gene targeting in higher plants [68]. Strong positive–negative selection associated with PCR screening has been successfully used to generate fertile transgenic rice plants with gene targeting [181, 182]. By this strategy, the frequency of gene targeting based on HR may reach approximately 2 % per surviving resistant callus [182].

Recently, two new technological breakthroughs have shown great potential application in improving the efficiency of gene targeting. One is zinc-finger nucleases (ZFNs). ZFNs can induce double-stranded breaks (DSBs) at the desired locus of the organism genome which can enhance the occurrence frequency of HR and improve the efficiency of gene targeting in higher plants [168, 186]. Gene targeting mediated by ZFNs has succeeded in maize [168] and tobacco [186]. Another breakthrough is TALENs (transcription activator-like effector nucleases). TALENs can also induce DSBs at a specific locus of DNA but seems to be superior to ZFNs due to more flexibility in design, no context dependence, and less off-target effects [33]. Gene targeting mediated by TALENs has succeeded in several model organisms including human pluripotent cells [55], rat [183], zebrafish [65, 152], and rice [96].

2.3 Chloroplast Transformation

Chloroplast transformation actually belongs to the technology of gene targeting also. The transgenes are usually delivered into the chloroplast by a direct method such as particle bombardment and then integrated into the chloroplast genome through HR of homologous sequence flanking transgenes [28]. There are two main advantages to implementing chloroplast transformation compared to nuclear transformation: higher expression efficiency due to high copy numbers of the chloroplast genome in the cell and less environmental risk due to inheritance in a maternal pattern. There are also many other advantages of chloroplast transformation. For instance, the inserted locus of the transgene is precise and predetermined resulting in elimination of the "position effect," because the integration occurs via HR; gene silencing, which is common for nuclear transformation, has never been reported in chloroplast transformation; and chloroplast genes are often arranged in operons that allow a promoter to drive multigenes in a polycistron, which facilitates multigene transformation [28]. The method of chloroplast transformation has been well established in dicotyledonous plants, especially for tobacco and tomato so far [148]. However, chloroplast transformation still has many technical difficulties for monocotyledons. There are few reports of chloroplast transformation for rice, and the transformation efficiency is very low [82, 92]. Moreover, the acquired chloroplast transgenic rice lines are heteroplasmic, and homoplasmy has not been achieved.

2.4 Transformation with a Large DNA Fragment

The method of transformation with a large DNA fragment is desirable for two main purposes: to accelerate the procedure of map-based cloning for complementation testing and to facilitate multigene transfer for pyramiding different traits or introducing a new metabolic pathway into plants [47, 99, 126]. However, transformation with a large DNA fragment such as >25 kb remains difficult, although efficient transformation methods have been established for many plant species [49]. Two special binary Ti vectors competent for the cloning and transfer of large DNA fragments have been reported: BIBAC (binary BAC) derived from bacterial artificial chromosome [47] and TAC (transformation-competent artificial chromosome) derived from P1 artificial chromosome [99]. The BIBAC vector has been confirmed to be capable of transferring at least 150 kb of foreign DNA into a plant [47], while the TAC vector is capable of transferring a DNA fragment of 80 kb [99]. Zhou et al. [229] reported that a 50-kb foreign DNA fragment was transferred into the rice genome via the TAC system. Genetic and PCR analyses in T_1 progeny confirmed that the inserted foreign DNA could be stably inherited. He et al. [49] recently reported that T-DNA from a 60-kb BIBAC clone

was successfully introduced into rice via *Agrobacterium*-mediated transformation, and molecular analysis confirmed that the integration and inheritance of the inserted fragment were stable in T_0, T_1, and T_2 generations of transgenic events. It is notable that *Agrobacterium* strains carrying an additional virulence helper plasmid with virG/virE are crucial for using the BIBAC system compared with *Agrobacterium*-mediated transformation using standard binary vectors. Another potential strategy for transferring a large DNA fragment is the artificial plant chromosome. There are two approaches to constructing an artificial plant chromosome. The first is the "bottom-up approach" in which artificial chromosomes containing essential elements including centromeric arrays, telomere repeats, and replication origins are assembled into one vector in vitro and then delivered into host cells to form artificial chromosomes. The second is the "top-down approach," in which artificial chromosomes are constructed by truncating endogenous chromosomes through the introduction of foreign DNA containing telomere repeats. Xu et al. [213] recently reported successful construction of artificial chromosomes in rice via the top-down approach. Although in principle the capacity of multigene engineering appears to have no limit, there are still many technological challenges for this strategy.

3 Insect-Resistant Rice

Rice stem borers, leaffolder (*Cnaphalocrocis medinalis*), and rice planthoppers are the most important rice pests worldwide and cause serious yield losses annually. Rice stem borers mainly include striped stem borer (*Chilo suppressalis*, SSB), yellow stem borer (*Tryporyza incertulas*, YSB), and pink stem borer (*Sesamia inferens*, PSB). Rice planthoppers include brown planthopper (*Nilaparvata lugens*, BPH), white-backed planthopper (*Sogatella furcifera*, WBPH), and small brown planthopper (*Laodelphax striatellus*, SBPH). Planthoppers not only suck the phloem sap but also spread serious viral disease as vectors of rice viruses.

Rice stem borers and leaffolders belong to the order Lepidoptera, which can be controlled effectively by *Bt* genes. *Bt* genes derived from *Bacillus thuringiensis* (Bt) are the most broadly used insecticidal genes and have specific insecticidal activities against species of the orders Lepidoptera, Coleoptera, Diptera, and Invertebrata (acarids, nematodes, and protozoa) [132]. *Bt* genes have been transferred and expressed in different crops including rice to develop insect-resistant crops. Bt cotton, corn, and potato have been commercially grown with huge economic and environmental benefits [71]. There have been many studies and evaluations of Bt rice in both the laboratory and the field since 1993 [2, 6, 18, 20, 23, 29, 38, 45, 83, 105, 106, 128, 144, 179, 188, 196, 203, 205, 218]. All results consistently confirmed that Bt rice was highly resistant to rice borers and leaffolder.

One concern in adopting Bt crops is that insects can evolve resistance against Bt crops, which will impair the crops' durability. Many Bt toxin-resistant insect strains have been selected in the laboratory or the greenhouse, and some can survive on Bt crops [7]. Moreover, at least some populations of three pest species have evolved resistance to Bt crops under natural field conditions in the USA [127]. Gene stacking is one strategy to delay the evolution of insect resistance and is based on the assumption that the likelihood that pests evolve simultaneous resistance to two Bt toxins decreases compared to a single Bt toxin if both toxins have different binding sites [147]. Yang et al. [216] developed two-toxin Bt rice lines by reciprocal hybridizations of four transgenic rice lines with different Bt genes, *cry1Ab* (1Ab), *cry1Ac* (1Ac), *cry1C** (1C), and *cry2A** (2A), in five combination patterns: 1Ab+1C, 1Ab+2A, 1Ac+1C, 1Ac+2A, and 1C+2A. Bioassay results in the laboratory showed that most two-toxin Bt rice lines exhibited higher insect resistance than those with a single *Bt* gene [216].

Bt rice was commercially approved in Iran in 2005 [70]. In 2009, China issued biosafety certificates for Bt rice variety Huihui No. 1 and its hybrid Bt Shanyou63 (http://www.moa.gov.cn/ztzl/zjyqwgz/spxx/201202/P020120203355472286958.

pdf). The first field experiments of Bt rice event T51-1, which is the original event of Huihui No. 1, were performed in China 1999 [188]. T51-1 contained a *cry1Ab*/*cry1Ac* fusion gene driven by the constitutively strong promoter rice *actin 1* promoter, and the estimated Cry1Ab/Cry1Ac fusion protein in T51-1 was a 20 ng/mg soluble protein. T51-1 showed high insect resistance under both natural and manual infestation conditions compared with the control Minghui63 in the field. Moreover, *Bt* Shanyou63, the hybrid of T51-1, showed the same yield potential as the non-transgenic hybrid control; and the eventual yield of *Bt* Shanyou63 was 28.9 % higher than the non-transgenic control, as no chemical pesticides were applied after transplanting (Tu et al. 2000). Production trials in China showed that adoption of Bt rice can increase yield by 6–9 %, drop pesticide use by 80 %, and reduce adverse health effects on farmers compared to adoption of non-transgenic rice varieties, implying huge socioeconomic and environmental benefits [61].

Planthoppers are the main rice sap-sucking pests, which are unfortunately not the target insects of Bt toxins. Snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) is a plant-derived gene that has been proved to have effects on rice sap-sucking pests. Studies demonstrated that GNA rice exhibited enhanced resistance against major rice sap-sucking insects including BPH [37, 120, 145], WBPH [121], SBPH [173], and rice green leafhopper (*Nephotettix cincticeps*, GLH) [37, 120]. The resistance conferred by GNA against rice sap-sucking insects is not comparable to that of Bt toxins against rice lepidopteran insects. GNA rice generally does not lead to high mortality of infested pests but instead has significantly adverse effects on their growth, development, and fecundity [120, 121, 145, 173]. Another plant lectin from garlic leaf (*Allium sativum* agglutinin from leaf, ASAL) was also tested in rice by Saha et al. [149]. ASAL rice exhibited enhanced resistance to BPH and GLH. In addition, since GLH is the vector of the rice viruses RTBV and rice tungro spherical virus (RTSV) that cause rice tungro disease, ASAL rice significantly reduced the infection incidence of rice tungro diseases when challenged by GLH infested with RTBV and RTSV [149].

The RNAi strategy provides new opportunities to control rice insects, especially for those that cannot be controlled by Bt toxins. This strategy has recently succeeded in developing plants resistant to cotton bollworm (*Helicoverpa armigera*) and western corn rootworm (*Diabrotica virgifera*) [9, 104]. Delivering dsRNA targeting BPH trehalose phosphate synthase gene in an artificial diet has been confirmed as effective in suppressing expression of the endogenous gene of BPH and significantly increasing nymph mortality [21]. A similar study was conducted by Li et al. [95], who found that feeding dsRNAs in an artificial diet targeting a BPH gene encoding vacuolar ATP synthase subunit E induced repression of the target gene of BPH, but the feeding did not cause a lethal phenotype. These two studies demonstrated that the RNAi mechanism exists in BPH and provide an important theoretical basis. However, it seems difficult to develop transgenic rice plants with satisfactory resistance using this strategy. DsRNAs targeting three endogenous genes (the hexose transporter gene *NlHT1*, the carboxypeptidase gene *Nlcar*, and the trypsin-like serine protease gene *Nltry*) of BPH were recently delivered through transgenic rice plants [221]. Molecular analysis showed that all three target genes in BPH were significantly suppressed, but a lethal phenotype was not observed, indicating that RNAi can be triggered in BPH by delivering dsRNA through transgenic plants [221]. A possible reason for the failure to obtain highly insect-resistant rice plants was probably that the selected target genes were not appropriate [221].

4 Disease-Resistant Rice

Crop disease resistance is regulated by two classes of genes: major resistance (*R*) genes that confer qualitative resistance and defense-responsive genes or quantitative trait loci (QTLs) that confer quantitative resistance (reviewed in [87]). Introduction of *R* genes into susceptible varieties via a transgenic approach is a convenient and

direct way to develop disease-resistant rice in case of the *R* genes that have been isolated. For instance, *Xa21*, a broad-spectrum *R* gene to bacterial blight, has been introduced into different rice varieties and the transgenic rice showed significantly enhanced resistance to multiple races of *Xanthomonas oryzae* pv. *oryzae* [32, 107, 189, 195]. Although defense-responsive genes or resistant QTLs generally provided comparatively minor but broad-spectrum disease resistance [87], many studies have shown that constitutive overexpression or suppression of quantitative resistance genes in rice can also confer effective resistance against bacterial and fungal pathogens. *Rir1b* is a defense-responsive gene that was identified by inoculation of rice with the non-host pathogen *Pseudomonas syringae* pv. *syringae*. Overexpression of *Rir1b* in rice conferred enhanced resistance to *Magnaporthe grisea* [156]. *pi21* is a recessive resistance QTL to rice blast. The suppression of the susceptible allele *Pi21* enhanced blast resistance in transgenic rice [41].

Expressing pathogen-derived protein elicitors in the host plant to induce the general defense response and systemic-acquired resistance (SAR) provides a promising strategy to develop broad-spectrum disease-resistant rice. Flagellin is a component of bacterial flagella which may act as a protein elicitor. Constitutive expression of flagellin from a phytopathogenic bacterium, *Acidovorax avenae* strain N1141, in rice triggered immune responses, and the transgenic rice plants showed enhanced resistance to *M. grisea* [176]. Shao et al. [161] reported that overexpression of the protein elicitor *harpin* derived from *Xoo* in rice conferred high nonspecific resistance to multiple *M. grisea* races.

Pathogenesis-related proteins (PRs) are a group of plant-encoded proteins that are exclusively induced by pathological or related situations, and many show antifungal activity in vitro [191]. Overexpression of PRs is a common strategy to develop disease-resistant rice. The PRs that have been attempted in transgenic rice include chitinase [31, 32, 86, 98, 107, 125, 130], β-1,3-glucanases [131], and thaumatin-like proteins [30]. Overexpression of the PRs individually or in combination can enhance resistance against *M. grisea* [31, 32, 98, 125, 131] and *Rhizoctonia solani* [86, 107, 130]. Similar to PRs, other antifungal proteins derived from plants or microorganisms have also been introduced into rice. Kanzaki et al. [80] reported that transgenic rice overexpressing a wasabi defensin gene conferred effective resistance against *M. grisea*. Constitutive expression of wheat-derived antimicrobial peptides puroindolines PINA and/or PINB in transgenic rice conferred significantly increased resistance against both *M. grisea* and *R. solani* [89]. Coca et al. [27] introduced an *Aspergillus giganteus* antifungal protein gene (*afp*) into rice, and the detached leaf infection assay showed that *afp* rice had enhanced resistance against *M. grisea*.

Rice is a natural host for 20 viruses and among them about 16 viruses may threaten rice yield [35]. The strategies to develop virus-resistant rice have been mainly based on the concept of pathogen-derived resistance [153], for which complete or partial viral genes are introduced into rice to interfere with one or more essential steps of the viral life cycle through a protein-mediated or RNA-mediated mechanism. In early stages, protein-mediated, especially viral coat protein (CP)-mediated, resistance was a general strategy to develop virus-resistant rice [48, 94, 160, 169].

RNAi plays an important role in natural resistance of host plants against viral infection [8]. Many studies have shown that RNAi-mediated resistance is practical and effective to develop virus-resistant rice. Initially, sense and antisense full-length or truncated viral genes were overexpressed in rice. For instance, enhanced viral resistance was acquired in transgenic rice by expressing a full-length or truncated RTSV replicase gene in both sense and antisense orientations [66]. Similarly, rice yellow mottle virus (RYMV)-resistant transgenic rice was obtained by expressing deleted or full-length RYMV RNA-dependent RNA polymerase (RdRp) genes [139]. Kouassi et al. [88] demonstrated that the expression of antisense or untranslatable CP mRNA in transgenic rice induced moderate resistance to RYMV while expressing wild-type CP or deleting the CP gene contrarily enhanced virus

infection, indicating that the enhanced viral resistance was related to an RNA-mediated mechanism [88].

Recently, new RNAi constructs have been used, in which partial segments of viral genes were constructed in an inverted repeat (IR) pattern. The new RNAi constructs with IR pattern can express double-stranded hairpin RNAs, which trigger RNAi more effectively. Tyagi et al. [190] introduced the IR RNAi construct of RTBV ORF VI into transgenic rice. Virus titer in the transgenic plants reduced significantly compared to the non-transgenic control when challenged by viruliferous GLH [190]. Shimizu et al. [162] introduced IR RNAi constructs of RDV-derived *Pns12* and *Pns4* genes in rice. The transgenic rice with *Pns12* RNAi construct showed strong resistance against RDV, while the resistance of rice plants with *Pns4* RNAi construct was less apparent. Shimizu et al. [163] introduced a set of IR RNAi constructs targeting different RSV genes into rice. The results showed that transgenic plants expressing RNAi constructs targeting RSV *pC3* and *pC4* genes were immune to RSV infection, but transgenic plants expressing RNAi constructs targeting RSV *pC2* and *p4* genes did not result in viral resistance. The results indicated that it is crucial to select appropriate target genes for using an RNAi strategy to develop virus-resistant rice [163].

5 Abiotic Stress-Tolerant Rice

Abiotic stresses comprising various adverse nonliving environmental conditions have become the greatest constraint to agricultural production worldwide, accounting for approximately 70 % of yield reductions annually [74]. As sessile organisms, plants have evolved complex regulatory networks to respond to and cope with environmental abiotic stresses. These regulatory networks are involved in signal perception and transduction, activation of transcription factors (TFs), and expression of downstream functional genes (reviewed in [42, 103, 124, 165]).

A general strategy to develop abiotic stress-tolerant crops is to manipulate the expression of the genes involved in abiotic stress response. The abiotic stress-responsive genes can be roughly classified into two groups: functional genes that directly protect the plant cell against stresses and regulatory genes, which function upstream of the abiotic stress-responsive networks [165].

In early studies, functional genes were commonly used as the target genes to develop abiotic stress-tolerant transgenic rice. These functional genes include late embryogenesis abundant (LEA) proteins [5, 24, 146, 208, 211], heat shock proteins (HSP [81, 119, 154]), Na^+/H^+ antiporters [19, 135, 193, 226, 227], and glycerol-3-phosphate acyltransferase (GPAT [3, 220]); catalytic enzymes synthesizing osmoprotectants including proline [171, 232], trehalose [43, 44, 72], glycinebetaine [115, 151, 155, 166, 172], and polyamines [16]; and detoxifying genes such as chloroplast glutamine synthetase [57], glutathione S-transferase [177], superoxide dismutase [140, 197], and catalase [118, 122]. Overexpression of these functional genes in rice has improved the tolerance of transgenic rice to various abiotic stresses (Table 24.1).

Recently, more studies have focused on manipulating regulatory genes to improve abiotic stress tolerance of transgenic rice. These regulatory genes include signal transducers such as Ca^{2+}-dependent protein kinases (CDPKs), calcineurin B-like protein-interacting protein kinases (CIPKs) and mitogen-activated protein kinases (MAPKs), TFs, and other stress-responsive genes. CDPKs, CIPKs, and MAPKs function upstream of signal transduction networks playing an important role in mediating various stress responses in eukaryotic organisms. Overexpression of the signal transducer genes can improve the tolerance of transgenic rice against various abiotic stresses. *OsCDPK7* is a cold- and salt-inducible rice *CDPK* [150]. Overexpression of *OsCDPK7* in rice enhanced the tolerance of transgenic rice seedlings to cold, salt, and drought stresses, and the extent of tolerance was consistent with expression level of *OsCDPK7* [150]. Xiang et al. [206] identified all of 30 rice CIPKs (*OsCIPK1–30*). Among them, 20 *OsCIPK* genes could be induced by at least one stress. Overexpression of three *CIPK* genes,

OsCIPK03, *OsCIPK12*, and *OsCIPK15*, in rice significantly enhanced the tolerances to cold, drought, and salt stresses, respectively, indicating that rice *CIPK* genes have diverse roles in different stress responses [206]. Overexpression of the rice *MAPK* gene *OsMAPK5* enhanced the tolerance of transgenic rice plants to drought, salt, and cold stresses, but resistance to fungal and bacterial pathogens decreased, indicating that *OsMAPK5* inversely regulates abiotic stress tolerance and disease resistance in rice [210]. *Dms1* was a drought hypersensitive T-DNA insertion mutant of rice putative MAPK kinase kinase (MAPKKK) gene. Transgenic rice plants overexpressing *DMS1* had significantly improved tolerance to drought stress at seedling stage [129].

TFs play an essential role in leading from the perception of abiotic stress to the expression of stress-responsive genes in the signal transduction network [67]. TFs are potential target genes to engineer abiotic stress-tolerant rice (reviewed in [67, 84]). NAC (NAM, ATAF, and CUC) is a large plant-specific TF family. A total of 140 members of the NAC family have been predicted in rice, and at least 18 of them were stress-inducible [73]. Studies proved that overexpression

Table 24.1 Summary of the recent transgenic trials to enhance abiotic stress tolerance of rice

Group	Gene function	Transgene	Source	Effect	References
Group I functional genes	Proline synthesis	*P5CS*	Moth bean	Drought- and salt-tolerant	[171, 232]
	Trehalose synthesis	*TPSP*	*Escherichia coli*	Drought- and salt-tolerant	[43, 72]
		OsTPP1	Rice	Salt- and cold-tolerant	[44]
	Glycine betaine synthesis	*CodA*	*Arthrobacter globiformis*	Drought-, salt-, and cold-tolerant	[115, 151, 155]
		COX	*Arthrobacter pascens*	Salt-tolerant	[172]
		CMO	*Spinach*	Salt- and temperature stress-tolerant	[166]
	Polyamine synthesis	*adc*	*Datura stramonium*	Drought-tolerant	[16]
	LEA protein	*HAV 1*	Barley	Drought- and salt-tolerant	[5, 146, 211]
		PMA80, *PMA1959*	Wheat	Drought- and salt-tolerant	[24]
		OsLEA3-1	Rice	Drought-tolerant	[208]
	HSP	*Hsp101*	*Arabidopsis*	Heat-tolerant	[81]
		sHSP17.7	Rice	Heat-tolerant and UV-B-resistant; drought-tolerant	[119, 154]
	GPAT	*GPAT*	*Arabidopsis*	Cold-tolerant	[220]
		SGPAT	Spinach	Cold-tolerant	[3]
	Detoxification genes	*GS2*	Rice	Salt-tolerant	[57]
		GST	Rice	Cold-tolerant	[177]
		MnSOD	Pea	Drought-tolerant	[197]
		Sod1	*Avicennia mayina*	Drought- and salt-tolerant	[140]
		katE	*Escherichia coli*	Salt-tolerant	[118, 122]
	Na^+/H^+ transporter	*AgNHX1*	*Atriplex gmelini*	Salt-tolerant	[135]
		SsNHX1	*Suaeda salsa*	Salt tolerant	[226]
		SsNHX1 and *AVP1*	*Suaeda salsa* and *Arabidopsis*	Salt-tolerant	[227]
		OsNHX1	Rice	Salt-tolerant	[19]
		PgNHX1	*Pennisetum glaucum*	Salt-tolerant	[193]

(continued)

Table 24.1 (continued)

Group	Gene function	Transgene	Source	Effect	References
Group II regulatory genes	Signal transducer	*OsCDPK7*	Rice	Drought-, salt-, and cold-tolerant	[150]
		OsMAPK5	Rice	Drought-, salt-, and cold-tolerant with increased susceptibility to bacterial and fungal pathogens	[210]
		OsCIPK03	Rice	Cold-tolerant	[206]
		OsCIPK12	Rice	Drought-tolerant	[206]
		OsCIPK15	Rice	Salt-tolerant	[206]
		Dms1	Rice	Drought-tolerant	[129]
	Transcription factor	*CBF3*	*Arabidopsis*	Drought-, salt-, and cold-tolerant	[133]
		ABF3	*Arabidopsis*	Drought-tolerant	[133]
		SNAC1	Rice	Drought- and salt-tolerant	[59]
		OsDREB1A,*1B*, *DREB1A*, *1B*, and *1C*	Rice and *Arabidopsis*	Drought-, salt-, and cold-tolerant with growth retardation	[69]
		OsNAC6	Rice	Drought- and salt-tolerant with growth retardation and yield reduction	[123]
		OsDREB1E, *OsDREB1G*, *and OsDREB2B*	Rice	Drought-tolerant	[20]
		ZFP177	Rice	Drought-tolerant	[63]
		SNAC2	Rice	Salt- and cold-tolerant	[60]
		OsDREB1F	Rice	Drought-, salt-, and cold-tolerant	[198]
		OsbZIP23	Rice	Drought- and salt-tolerant	[207]
		ZFP252	Rice	Drought- and salt-tolerant	[212]
		OsWRKY11	Rice	Drought- and heat-tolerant	[204]
		AP37	Rice	Drought- and salt-tolerant	[134]
		AP59	Rice	Drought-, salt-, and cold-tolerant	[134]
		DST	Rice	Drought- and salt-tolerant	[64]
		OsNAC045	Rice	Drought- and salt-tolerant	[228]
		OsNAC10	Rice	Drought-tolerant	[73]
	Stress-responsive genes	*OsCOIN*	Rice	Drought-, salt-, and cold-tolerant	[100]
		OCPI1	Rice	Drought-tolerant	[62]
		OsiSAP8	Rice	Drought-, salt-, and cold-tolerant	[78]
		OsSKIPa	Rice	Drought- and salt-tolerant	[58]
		OsMT1a	Rice	Drought-tolerant	[215]

of some rice *NAC* genes in rice could enhance the resistance to different abiotic stresses. The transgenic rice overexpressing *SNAC1* showed enhanced drought and salt tolerance at the vegetative stage. The seed setting rate of *SNAC1* rice increased 22–34 % under drought stress at the reproductive stage in field conditions compared to the wild-type control [59]. Overexpression of *OsNAC6* in rice resulted in enhanced tolerance to drought and salt stresses [123]. However, transgenic rice plants overexpressing *OsNAC6* constitutively also exhibited adverse symptoms such as growth retardation and yield reduction compared to the control [123]. Transgenic rice overexpressing *SNAC2* exhibited enhanced tolerance to drought, salt, and cold stresses at the seedling stage [60]. Overexpression of *OsNAC045* in transgenic rice significantly increased the tolerance

to drought and salt stresses at the seedling stage [228]. *OsNAC10* was overexpressed in rice under the control of both the constitutive and the root-specific promoters by Jeong et al. [73]. Field evaluation showed that *OsNAC10* rice using the root-specific promoter exhibited higher grain yield under both drought and normal conditions compared to the controls. Moreover, *OsNAC10* rice with root-specific promoter had significantly superior field performance compared to *OsNAC10* rice with a constitutive promoter [73].

DREB/CBF (DRE-binding protein/C-repeat binding factor) are a class of TFs that interact with cis-acting element DRE/CRT (dehydration-responsive element/C-repeat), which exists in the promoter regions of many stress-responsive genes [164]. DREB/CBF contains two subclasses: DREB1/CBF and DREB2 [164]. Oh et al. [133] introduced *Arabidopsis*-derived TFs CBF3/DREB1A and ABF3 (ABA-responsive element binding factor 3) in rice and found that transgenic rice overexpressing CBF3/DREB1A exhibited increased tolerance to drought and salt stresses and slightly increased tolerance to cold, while overexpression of ABF3 increased drought tolerance alone. Ito et al. [69] reported that overexpression of three *Arabidopsis*-derived DREB1 genes (*DREB1A*, *DREB1B*, and *DREB1C*) and two rice orthologs of *DREB1A*, *OsDREB1A*, and *OsDREB1B*, in rice enhanced the tolerance of transgenic rice to drought, salt, and cold stresses but caused growth stunting under normal conditions. *OsDREB1F* was a salt-induced DREB/CBF gene isolated from upland rice via subtractive suppression hybridization (SSH). Overexpression of *OsDREB1F* in rice improved the tolerance of transgenic rice to drought, salt, and cold stresses [198]. Chen et al. [20] isolated three rice DREB/CBF genes *OsDREB1E*, *OsDREB1G*, and *OsDREB2B*—overexpression of these three in rice improved the tolerance to drought stress by different degrees [20].

Other TF genes including *ZFP252* [212], *ZFP177* [63], *OsbZIP23* [207], *OsWRKY11* [204], *AP37* and *AP59* [134], and *DST* [64] and abiotic stress-responsive genes such as *OsCOIN* [100], *OsiSAP8* [78], *OsMT1a* [215], *OCPI1* [62], and *OsSKIPa* [58] have also been tested in transgenic rice. The origins and effects of these genes are listed in Table 24.1.

Manipulation of regulatory genes can change the expression levels of a great number of downstream abiotic stress-responsive genes and is considered to be more promising than functional genes with simple functional modes [67]. In most previous studies, the target genes were overexpressed constitutively. However, constitutive overexpression of regulatory genes improves abiotic stress tolerance but sometimes also causes adverse effects [69, 123]. Using an appropriate promoter such as the tissue-specific or stress-inducible promoter may solve this problem [54]. Tissue-specific or inducible overexpression of regulatory genes is probably more effective than constitutive overexpression. For instance, root-specific overexpression of *OsNAC10* in rice increased the grain yield of transgenic rice compared to controls under both drought and normal conditions in the field, while constitutive expression of the same gene in rice showed no difference in grain yield to controls under the same conditions [73].

6 High Nutrient-Use Efficiency Rice

6.1 Nitrogen

Nitrogen (N) is an essential nutrient that plants require in the greatest quantity and is also one of major limiting factors in plant production. Nitrogenous fertilizers represent the major cost of plant production, as it is estimated that 85–90 million tonnes of nitrogenous fertilizers are added annually to the soil to enhance yield performance of plants worldwide [46]. However, approximately 50–70 % of this applied N runs off from the plant–soil system [46], consequently polluting soil and water [108]. Therefore, it is very important to improve the nitrogen use efficiency (NUE) of plants in order to lower N fertilizer inputs.

N uptake, assimilation, and remobilization in the plant have been well documented (reviewed

in [79]). Manipulating the expression of those genes to enhance the capacity of N uptake, assimilation, and remobilization is a common strategy to develop transgenic rice with high NUE. Studies showed that overexpression of ammonium (NH_4^+) transporters have not acquired satisfactory efficacy. Kumar et al. [91] found that the biomass of transgenic lines overexpressing *OsATM1*;*1* reduced or had no statistical difference compared to the wild-type control at both 2 and 10 μM external NH_4^+ concentrations, although $^{13}NH_4^+$ influx in some transgenic lines was higher than the wild type at 10 μM external NH_4^+ concentration. The study of Hoque et al. [56] showed similar results: the biomass of *OsAMT1*;*1* overexpressing transgenic rice significantly decreased at early growth stages with increased NH_4^+ uptake and accumulation at high NH_4^+ concentration (2 mM) compared to the wild-type control. The decrease of biomass was probably because NH_4^+ assimilation could not match the enhanced NH_4^+ uptake of roots due to overexpressing *OsAMT1*;*1* in transgenic rice [56].

Cai et al. [15] overexpressed two endogenous *GS* genes (*GS1*;*1* and *GS1*;*2*) and a bacterial *GS* gene (*glnA*) in rice. They found that all GS-overexpressed (including *GS1*;*1*, *G1*;*2* and *glnA*) transgenic plants showed significantly increased total amino acids and total N content in the whole plant compared to the wild-type control under both low and high N conditions. However, both grain yield and total amino acids in seeds of all GS-overexpressed rice plants decreased compared to the wild-type control under field conditions with N deficit stress. Brauera et al. [13] evaluated the NUE of transgenic rice overexpressing rice *GS1*;*2* under different nitrate conditions and growth environments compared to both the azygous and wild-type controls. The harvest index, N harvest index, and utilization N use efficiency (NUE) of transgenic rice lines increased under two certain experimental conditions compared to the azygous control. However, *GS1*;*2* overexpressing rice lines did not show higher NUE under limiting N compared to non-limiting N conditions, indicating that *GS1*;*2* overexpressing rice could not use less N under field conditions [13].

Yamaya et al. [214] demonstrated that overexpression of a rice NADH-dependent glutamate synthase (NADH-GOGAT) in an *indica* cultivar Kasalath significantly increased grain weight (up to 80 %) compared to the non-transgenic control, indicating an important role of NADH-GOGAT for N utilization and grain-filling in rice. Abiko et al. [1] introduced a fungus-derived NH_4^+ assimilation gene NADP(H)-dependent glutamate dehydrogenase (*gdhA*) in rice. The transgenic rice had increased biomass and N content compared to the control under N sufficient conditions, but no significant difference under N deficit condition. Under field conditions, *gdhA* rice exhibited higher grain yield compared to the control.

Aminotransferases are key enzymes for amino acid synthesis. Shrawat et al. [167] reported that tissue-specific expression of a barley alanine aminotransferase (*AlaAT*) cDNA in rice roots significantly increased the biomass and grain yield compared to the non-transgenic control. In addition, the Gln and total N content of *AlaAT* rice plants also increased, indicating enhanced N uptake efficiency. Zhou et al. [231] overexpressed all of three rice aspartate aminotransferase (*ATT*) genes (*OsAAT1–3*) and a bacterial *AAT* gene (*EcAAT*) in rice. None of the transgenic rice plants overexpressing these four genes showed significant differences in main agronomic traits and yield from the wild-type control.

Besides the genes involved in N uptake, assimilation, and remobilization, N-responsive genes have also been used as target genes to engineer high NUE rice. *OsENOD93-1* is an early nodulin gene responding to external nitrate concentration changes (either induction or reduction). Overexpression of *OsENOD93-1* in rice resulted in increased shoot biomass, seed yield, and higher total amino acid and total N in roots compared to the wild-type control [10]. Asano et al., (2010) demonstrated that transgenic rice overexpressing the full-length cDNA of a rice calcium-dependent protein kinase (*CDPK*) *OsCPK12* was more tolerant under low N stress than the control. The shoot biomass and total N content of transgenic lines were significantly increased compared to the control [4].

6.2 Phosphorus Use Efficiency

Phosphorus (P) is the most essential macroelement for crop production after N and plays a very important role in many metabolic pathways. The absolute P amount in the soil is comparatively abundant, but the P available to plants is generally deficient due to its low solubility and high adsorptive capacity [143]. Therefore, improving P acquisition and use efficiency of crops is critical for sustainable and environment-friendly agriculture.

Phosphate transporters (PTs) play an important role in the acquisition of P through the roots under low external P concentrations. Manipulating the expression of *PT* genes is therefore a reasonable strategy to enhance the P absorption of plants. Seo et al. [159] overexpressed a rice *PT* gene *OsPT1* in rice. Under both normal and P-null conditions, transgenic rice plants overexpressing *OsPT1* accumulated almost twice as much inorganic P (Pi) TF in the shoots, with more tillers and better root development compared to the wild-type control. However, transgenic rice plants overexpressing *OsPT1* were 30 % shorter than the wild-type control, which was attributed to the comparative deficiency of N and potassium as they were not concomitantly increased with the enhanced P acquisition [159]. Liu et al. [101] demonstrated that overexpression of OsPT2, a low-affinity PT, in rice resulted in increased shoot P content of the transgenic plants with obvious Pi toxicity symptoms: leaf chlorosis or necrosis and growth retardation under Pi-sufficient conditions. Similar to OsPT2, overexpression of OsPT8 in rice also resulted in excessive shoot P accumulation with Pi toxic phenotypes under Pi-sufficient conditions [75].

Besides *PT* genes, regulator genes involved in the Pi-signaling pathway have also been tested in transgenic rice. OsPTF1 is a P-deficiency-responsive TF identified from Kasalath, a P-use efficient *indica* rice landrace [219]. Overexpression of OsPTF1 in Nipponbare, a low-P sensitive *japonica* rice variety, significantly enhanced tillering ability, root and shoot biomass, and P content of transgenic rice under P-deficient conditions [219]. OsPHR1 and OsPHR2 are two rice homologs of AtPHR1, a previously identified *Arabidopsis* TF which plays a central role in the Pi-signaling pathway [230]. The expression of *OsPHR1* and *OsPHR2* genes was both overexpressed and depressed in rice, respectively [230], and only transgenic rice plants overexpressing *OsPHR2* exhibited phenotypes different from the wild-type control. *OsPHR2* overexpressing transgenic rice accumulated higher Pi in shoots compared to the wild-type control under Pi-sufficient conditions with Pi toxicity symptoms [230]. OsPHF1 is the rice homolog of AtPHF1 that regulates the plasma membrane localization of high-affinity PT, PHT1;1 in *Arabidopsis* [22]. Overexpression of *OsPHF1* caused excess P accumulation in both shoots and roots with Pi toxicity phenotypes under Pi-supplied condition [22].

In most studies, overexpression of *PT* genes or regulator genes involved in the Pi-signaling pathway results in Pi accumulation in transgenic rice plants, indicating enhanced Pi acquisition capacity. However, Pi toxicity phenotypes are also produced under Pi-sufficient conditions in many studies. In most previous studies, transgenes were generally overexpressed, driven by constitutive promoters. Therefore, expressing the transgenes under the control of inducible or tissue-specific promoters would probably mitigate the Pi toxicity phenotypes. Moreover, overexpression of multiple genes involved in the Pi metabolism pathway to enhance the overall levels of Pi acquisition, transportation, and localization would probably improve the Pi metabolism of transgenic rice and mitigate the Pi toxicity phenotypes.

7 Quality

Rice quality generally consists of four main categories: processing quality, appearance quality, cooking and eating quality, and nutritional quality [222]. Transgenic approaches have been applied mainly to improve the nutritional quality of rice. As one of the most important staple crops, rice is a main source of energy for rice-consuming populations. However, rice lacks some important micronutrients such as vitamin A, iron, and zinc [112]. This results in micronutrient malnutrition

prevailing in rice-consuming populations especially in the developing countries of South and Southeast Asia, where their dietary food mostly consists of rice. Micronutrient malnutrition causes various health issues and higher child mortality [113].

Golden rice (GR) is the transgenic rice with enhanced β-carotene [217]. β-carotene is the precursor of vitamin A and can be easily converted to vitamin A in the human body. The carotenoid biosynthesis pathway is naturally nonexistent in rice endosperm but was established by introducing two foreign genes, phytoene synthase gene (*psy*) from daffodil (*Narcissus pseudonarcissus*) and phytoene desaturase (*crtI*) from *Erwinia uredovora*, into rice [217]. In endosperm of the best GR line, there was 1.6 μg/g of accumulated carotenoids [217], which was a comparatively low content of β-carotene compared to the recommended daily allowance of vitamin A. An improved version of GR, also referred to as GR2, was developed in 2005 [137]. Carotenoid content in GR2 was increased by approximately 23 times compared to the original GR by replacing the daffodil *psy* gene with a maize-derived ortholog [137]. GR will hopefully be commercially available by 2013 [71], which will relieve the health issues caused by vitamin A deficiency.

The first case of iron-biofortified transgenic rice was reported by [230]. The iron content in the seed of the transgenic rice was increased threefold compared to the non-transgenic control by endosperm-specific overexpression of a soybean iron storage protein gene *ferritin* [230]. A similar strategy was attempted by Vasconcelos et al. [192] and Qu et al. [142]. Vasconcelos et al. [192] found that accumulation of ferritin protein in rice endosperm could increase both iron and zinc content in the polished rice grain. The study of Qu et al. [142] showed that iron accumulation did not parallel the overexpression of *ferritin* in rice seed, implying that higher iron content is unlikely to be achieved by further improving the expressional level of ferritin protein in seed of transgenic rice.

Lucca et al. [102] attempted an integrated strategy: to simultaneously improve the iron content of rice seeds and iron bioavailability. Up to a twofold increase of iron content in seeds of transgenic rice was achieved by endosperm-specific overexpression of the common bean (*Phaseolus vulgaris*) *ferritin* gene. Meanwhile, to improve iron bioavailability, a thermotolerant phytase from *Aspergillus fumigates* was endosperm-specifically overexpressed and an endogenous cysteine-rich metallothionein-like protein was overexpressed [102]. Phytase can degrade phytic acid that inhibits the absorption of iron as a strong chelator of minerals, while cysteine or cysteine-containing peptides can significantly enhance iron uptake in humans.

Mugineic acid family phytosiderophores (MAs) play a very important role in iron uptake from the soil and iron transport within the plant in graminaceous plants. Barley genes involving in MA biosynthesis (i.e., *HvNAS1*, *HvNAAT-A*, and *HvNAAT-B* or *IDS3*) were introduced into rice [109]. Limited increase of iron content (1.4 times) was found in polished rice seeds of the *IDS3* rice plants compared to the non-transgenic control [109].

Nicotianamine (NA) is a chelator of metal cations such those of iron and zinc. Overexpression of nicotianamine synthase (NAS) can result in higher accumulation of nicotianamine and improve iron and zinc transport and translocation in transgenic plants. The barley *NAS* gene *HvNAS1* has been introduced into rice [110]. Iron and zinc content in polished seeds of *HvNAS1* rice increased more than three- and twofold, respectively [110]. Three endogenous *NAS* genes, *OsNAS1*, *OsNAS2*, and *OsNAS3*, have been constitutively overexpressed respectively in rice [76]. All three populations (*OsNAS1*, *OsNAS2*, and *OsNAS3*) of transgenic rice had increased iron and zinc contents in the seeds compared with the control. The highest iron content of polished seeds was found in *OsNAS2* rice line with 19 μg/g, representing up to a fourfold increase compared to the control. The zinc concentration in polished seeds of *OsNAS2* rice also increased twofold [76]. This is the highest iron biofortification achieved via a single gene strategy so far, indicating the enormous potential of *OsNAS2* in breeding iron-biofortified rice. A more than sixfold increase of iron content in polished seeds

of transgenic rice was achieved by synergistic action of *Arabidopsis* NAS (constitutive overexpression) and common bean ferritin (endosperm-specific overexpression [202]).

8 Yield

Constant growth of the world population has been putting huge pressure on rice yield. It is estimated that population growth in Asia requires a 60 % increase of rice production by 2050 [77]. Developing C_4 rice to enhance rice photosynthetic capacity is considered a possible strategy to substantially increase rice yield and support the projected population growth [77].

Higher plants can be divided into three groups: C_3, C_4, and crassulacean acid metabolism (CAM) plants according to the initial photosynthates of CO_2 in the carbon assimilation pathway during photosynthesis. C_4 plants evolved from C_3 plants but have higher photosynthetic capacity and higher water-use efficiency and NUE than C_3 plants due to their special CO_2 assimilation and concentration mechanism (reviewed in [50, 93, 111]). Unfortunately, many agronomically important crops such as rice, wheat, barley, and soybean are C_3 plants.

The first attempt to produce C_4 rice was conducted by Ku et al. [90]. In their study, the maize phosphoenolpyruvate carboxylase (PEPC), which catalyzes the initial fixation of CO_2 in C_4 plants, was introduced into rice. The transgenic rice plants exhibited reduced O_2 inhibition of photosynthesis compared to the non-transgenic control [90]. However, it was later shown that the reduced O_2 inhibition of photosynthesis in *PEPC* rice was not caused by the participation of the foreign PEPC in photosynthetic CO_2 fixation as supposed initially but was caused by lower net CO_2 assimilation rate [40].

Other C_4 cycle-related genes have also been introduced individually into rice, including pyruvate orthophosphate dikinase (*PPDK* [39]), phosphoenolpyruvate carboxykinase (*PEP-CK* [174]), NADP-malic enzyme (*ME* [178, 187]), and NADP-malate dehydrogenase (*MDH* [180]). However, overexpression of single C_4 cycle-related genes in rice generally causes null or even negative effects on photosynthesis and plant growth (reviewed in [114, 180]).

A more complex strategy was attempted, in which several C_4 cycle-related enzymes were introduced into rice in combination to establish a single-cell C_4 photosynthetic pathway mimicking C_4 photosynthesis of some aquatic plants [114, 180]. Differing from classical terrestrial C_4 plants, some aquatic plants have a comparatively simple C_4 photosynthetic pathway that accomplishes C_4 photosynthesis in a single cell without compartmentation and chloroplast differentiation [12]. In the single-celled type of C_4 rice, the initial CO_2 fixation was supposed to be catalyzed by *PEPC* in the cytosol to form C_4 acid, and then the resultant C_4 acid was transferred into the chloroplast and decarboxylated there. Single-celled C_4 photosynthetic pathways consisting of two enzymes PEPC and PEP-CK [175] or four enzymes PEPC, PPDK, NADP-MDH, and NADP-ME [180] have been tested in rice, respectively, but the results of both studies were disappointing. PEPC/PEP-CK rice showed reduced chlorophyll concentration and abnormal thylakoid membranes compared with the non-transgenic control [175]. Transgenic rice plants pyramiding four enzymes—PEPC, PPDK, NADP-MDH, and NADP-ME—only exhibited slightly improved photosynthesis accompanied with slight but reproducible plant stunting [180].

Recently, a novel strategy of two-celled C_4 rice that mimics the classic two-celled C_4 photosynthetic pathway in terrestrial C_4 plants was proposed, in which the classical enzymes of the NADP-ME-type C_4 cycle were overexpressed in rice in a cell-specific manner and meanwhile endogenous RuBisCO and glycine decarboxylase genes were cell-specifically down-regulated in mesophyll cells [77]. This novel strategy has not been attempted previously. It is difficult to determine whether this strategy will be effective because no detailed data have yet been presented [77].

9 Prospects

Significant achievements have been made in transgenic rice research in the past 2 decades. Not only have mature transformation systems been established, but a great many transgenic rice materials with various improved traits have been

acquired as well. However, there are still many needs to be met for further development of many aspects of transgenic rice.

From a technology perspective, a highly efficient transformation system for *japonica* rice has been established, but many *indica* rice varieties are still obstinate. The N_6 medium that is very amenable for the tissue culture of *japonica* varieties is not suitable for most *indica* varieties. Meanwhile, comparatively lower transformation efficiency of *indica* rice is also a restriction factor. Hiei and Komari's [52] new protocol is adaptable for *indica* rice varieties but requires immature embryos as the explants. Collection and isolation of immature embryos are time-consuming and limited by the growth season. A highly efficient transformation system using mature seed for *indica* rice would still be valuable. Special transformation technologies such as gene targeting, chloroplast transformation, and DNA transfer with large fragments have great potential application in rice transformation; however, they have not been widely applied due to some technical difficulties.

The RNAi strategy provided new opportunities to engineer insect-resistant rice, especially for controlling rice planthoppers which are not sensitive to Bt toxins. Feeding tests in vitro have showed that dsRNA can suppress the endogenous gene expression of BPH and cause increased nymph mortality. However, highly BPH-resistant rice expressing BPH-derived dsRNAs has not been acquired, implying that some key technical issues must be addressed in future. These technical issues probably include how to increase dsRNA concentrations in the phloem of transgenic rice and how to select and determine effective target genes.

Engineering complex multigenic traits including abiotic stress tolerance, nutrient-use efficiency, and C_4 rice currently remains an enormous challenge. Although some transgenic rice showed improved abiotic stress tolerance or nutrient-use efficiency in certain experimental conditions, none have showed satisfactory performance under complex field conditions. Attempts at developing C_4 rice over the past 10 years have not achieved encouraging results. A new strategy to produce two-celled C_4 rice has therefore been proposed recently, but only very preliminary results have been acquired so far. More fundamental research to further understanding biological mechanisms and identifying more functional genes involved in these multigenic traits are still necessary to improve these traits by genetic manipulation in the future.

GR and iron-biofortified rice are produced mainly for poor people. Chronic noncommunicable diseases including cancers, heart diseases, type 2 diabetes, and obesity are major human health issues of all populations. Transgenic plants have showed the great potential application in promoting human health and reducing the risk of chronic disease. For example, a special transgenic purple tomato with high anthocyanin extended the life span of cancer-susceptible mice by approximately 30 % when supplemented in their diet [14]. As an important staple food crop, it would be highly desirable if transgenic rice with improved nutrient quality can benefit all human populations.

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Perspective Rice 2020 Revised: An Urgent Call to Mobilize and Coordinate Rice Functional Genomics Research Worldwide

Rod A. Wing and Qifa Zhang

In 2008 Qifa Zhang and colleagues from China proposed "Rice 2020" as an international call to coordinate research activities aimed at functionally characterizing the rice genome with the eventual goal of creating new varieties of rice with greatly increased yield potential but with less of an environmental footprint—the so-called "green super rice" varieties [5, 6].

Such a unified effort is extremely important as the world population will increase to nine billion inhabitants or more in under 40 years, and thus we must do all that we can to ensure a safe and secure food supply for generations to come.

The genus *Oryza*, of which two cultivated species (*O. sativa* & *O. glaberrima*) of rice are members [1], will play a key role in our efforts to lead the next green revolution. Rice already feeds half the world and it is that half that is predicted to double in size in less than 40 years.

The seven main themes of the Rice 2020 International Rice Functional Genomics Project were to (1) develop enabling tools and genetic resources; (2) assign biological functions to every annotated gene; (3) develop systems-wide gene expression profiles, epigenomes, and regulatory networks; (4) perform global analyses of the proteome and protein–protein interactions; (5) enhance bioinformatics platforms for data management and exchange and sharing of information; (6) establish a tool kit for high-throughput knowledge-based rice breeding; and (7) understand and exploit natural variation of *O. sativa* and its wild relatives.

Rather than rehash these themes, many of which are addressed and updated in this book, we will attempt to update the current state of the art of each theme from our perspective and propose a new paradigm shift as to how we can better coordinate our activities to achieve our common goals.

Rice 2020 [6]: Update and Perspectives

Theme 1 proposed the "*Development of Enabling Tools and Genetic Resources for an International Community of Scientists to Conduct Functional Genomics Research in Rice*" and emphasized three main areas: i.e., (a) insertion collections, (b) full-length cDNA collections, and (c) artificial micro-RNA (amiRNA collections).

Regarding insertion collections (Theme 1a), Rice 2020 summarized that the rice community had already generated sufficient mutant resources (>500,000) and the generation of flanking sequence tags (FSTs) to determine the precise location of each insertion should be prioritized. It is clear that the generation of a complete set of

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genetically mapped FSTs is critically needed and resources need to be dedicated toward completing this objective as soon as possible.

Critical problem: Unfortunately, many practical and logistical problems are associated with the development of such important mutant resources which include maintenance of viable seed stocks and the ability to share and distribute large knockout collections across international borders without restrictions (e.g., quarantine restrictions, material transfer agreements [MTAs]).

Ideal solution: In an ideal world it would be beneficial to have MTAs lifted and all mutant resources deposited in 5–10 locations around the world. The collections would be grown out on an annual basis for collective phenotyping and tissue collection. This would permit rapid distribution and phenotyping of the world collection of knockout mutants in a timely, efficient, and coordinated manner.

Theme 1b: Full-length cDNA collections. Full-length cDNA collections are an elegant resource and are very useful for gene and protein expression work. However, as sequencing and cDNA synthesis costs continue to plummet, it is now realistic to predict that the rice community is capable of generating full-length quantitative cDNA expression data from every developmental stage, tissue type, and cell type of a growing rice plant, from seedling to flower and seeds, under a plethora normal, and key biotic and abiotic stress conditions.

Such experimental conditions and tissue samples would also permit coordinated in-depth explorations of rice proteomes, metabolomes, methylomes, and on-omes.

Theme 1c: Artificial micro-RNA (amiRNA collections). AmiRNA collections for all rice genes should be given a high priority. It is important that one to two research groups be given this assignment and that such resources are deposited in the public domain. In turn, these collections should be systematically transformed into rice to begin to knock out all genes and gene families (see below).

Themes 2–4 and 7 can be grouped together into one general theme.

Briefly, Theme 2 proposed the "*Assignment of Biological Functions to Every Annotated Gene*" with two aims: (1) systematic phenotyping and characterization of mutants, and (2) systematic characterization of gene families.

Theme 3 proposed a "*Systems-Wide Epigenomes*, *Gene Expression Profiles and Regulatory Networks*" with three aims: (1) comprehensive cell- or tissue-specific epigenomes and transcriptomes for selected developmental stages, abiotic and/or biotic conditions; (2) identification of regulatory elements based on the epigenetic profiles and transcriptomes; and (3) systematic characterization of regulatory hierarchy of genome expression, its relationship to epigenomes during development and responses to various environmental changes, and their effects on growth and development.

Theme 4 proposed a "*Global Analyses of the Proteome and Protein–Protein Interactions*" with two aims: (1) tissue-specific proteomes of selected developmental stages and under selected defense and stress conditions; and (2) an experimentally defining comprehensive protein–protein interaction network.

Theme 7 proposed the "*Establishment of the Toolkit for High-Throughput Knowledge-Based Rice Breeding*".

Ideal solution: With the advent of glasshouse, field, and airborne-level high-tech phenotyping capabilities, coupled with advanced computational resources (e.g., iPlant), it is now conceivable that our community could interrogate the majority of all rice mutants, elite cultivars, land races, and wild relatives of rice in a detail (see Theme 1b) never dreamed of 3–5 years ago. Such datasets would allow breeders to "design" the ideal rice plant and model plant growth/yield under every relevant condition to achieve maximum yield and quality and minimum environmental impact.

Theme 5 proposed the establishment of a comprehensive understanding of "*Natural variation of O. sativa and its* (*wild*) *relatives*".

Cataloging SNP and structural variation across all rice cultivars, land races and its wild relatives will provide an unprecedented resource for crop improvement, association mapping,

genomic selection, and ecological and evolutionary research. Many labs around the world have re-sequenced 10–1,000 s of accessions, and most of this data is or will be made publically available for varied analyses. It should, however, be emphasized that It is critical that all biological resources used to generate these important datasets are made publically available across international borders, without restrictions, in order to take full advantage of this emerging resource.

In addition to the datasets above it is important to stress the need to better exploit the wild relatives of rice. As discussed in the *Oryza* Genetics and Genomes chapter [3], there will be a complete set of reference genome sequences available for all diploid *Oryza* species (18 species) by 2014. The wild relatives of rice contain a virtually untapped reservoir of genes that can be used for crop improvement and these International-*Oryza* Map Alignment Project (I-OMAP) resources will greatly facilitate their integration into the mainstream of rice functional genomics.

Theme 6: Bioinformatics, data management, and exchange and sharing of information.

Phenotyping (at multiple levels) and genotyping the genus *Oryza*, as discussed above, will yield an unprecedented amount of data for the world's most important food crop. The rice and agricultural community (in general) must position itself to be able to store, manage, share, and make use of such large and complex datasets to address the grand challenge questions of our times to achieve the ultimate goal of creating a sustainable world food supply.

$9 Billion to Help Solve the 9 Billion-People Question

The Rice 2020 [6] and Green Super Rice [5] initiatives are bold concepts that need to be taken seriously and embraced by the agricultural research community. In reality, our community has less than 25 years to solve the 9 Billion-People Question (9BPQ) in order to supply farmers with the seed necessary to feed the world. The challenge is enormous, and we must act now, and at a scale never before seen, if we are to achieve the food and security needs of our children and beyond.

Critical problem: Although enormous strides have been achieved at all levels in plant biology in recent years, there has been a major lack of coordination between countries in terms of conceiving a unified plan aimed at solving world hunger. One exception to this trend was when ten nations joined hands (and brains) in 1998 to generate the highest quality and publically available reference genome sequence for any crop plant—rice [4]. The funding agencies of these nations wisely recognized the importance of generating a gold standard genome sequence for the world's most important food crop which has already had an enormous impact on plant biology and breeding in general.

Even though the rice genome has been utilized extensively to understand many aspects of rice biology, a coordinated effort to systematically understand its genome (and accessions, cultivars, land races, and wild relatives) to be able to design green super rice varieties adapted to all rice growing regions is completely lacking. In fact, rather than working as a community to help solve the 9BPQ, rice scientists are competing with one another to, e.g., clone the next gene or QTL, write the best paper, get the next grant: a process that is repeated over and over again, worldwide. Competitive science is extremely important and critical in the discovery process, but given our timeline on the world stage, research on rice must enter a new and bold era of worldwide coordinated basic, translational, and applied research if we are to solve the 9BPQ in 25 years.

Proposed solution: $9 billion to help solve the 9BPQ.

To achieve our goals of creating new green super rice varieties that not only meet the caloric and nutritional needs of our society by 2050 but also protect our environment with less resources spent on production, we propose the "$9 billion to help solve the 9 billion people question" concept.

To begin, nine billion USD is not a lot of money in the scheme of the world economy. In fact this dollar figure equates to only 1 dollar per person by 2050—a deal if one considers what

could be achieved to save our planet and reduce the risk of economic and global border instability. Nine billion dollars is not a funding scale that most plant biologists are used to talking about. To put this dollar figure into perspective, it costs three billion USD to send Curiosity to Mars which landed in the summer of 2012 [2]. One could argue that NASA's Mars Science Laboratory will help save our planet, but in the very very long term. We have only 25 years to solve the 9BPQ and must act now if we want future generations to reap the benefits of Curiosity and other future planetary missions.

Our proposed solution is relatively simple but will require open and in-depth discussions from various stakeholder groups to work out the details and specifics. It could also serve as a model for other agriculture initiatives. The initial concept is to raise 2–3 billion dollars that would be used to establish 5–6 International Green Super Crop Science and Technology Centers (IGSCSTCs). Half of the money would come from philanthropic organizations and individual donors, and the remaining would come from national funding agencies as dollar-for-dollar matching funds. For example, one could imagine the establishment of six IGSCSTCs in Africa, India, South America, China, the European Union, and North America. Regional funding agencies would each provide 250 million USD for a total of 1.5 billion USD. This amount would be matched by philanthropic organizations for a total of three billion USD. The IGSCSTCs would be built and partially staffed within 2 years of raising the funds. If these funds could be secured by January 2015, the STCs would be completed and staffed by 2017. That will give us about 20 years to achieve our common goal. The remaining six billion USD would be used to support the 6 STCs at an average ~46 M USD/year/STC and would come from a combination of National funding agencies (e.g., the United Nations) and philanthropic organizations.

The goal of the STCs would be to perform coordinated basic, translational, and applied research aimed at creating green super crops with an emphasis on rice. The STCs would house state of the art research facilities, and would include regional greenhouse, field, and aerial phenotyping centers that would phenotype regional accessions as well as a common set of accessions/populations that would be used to obtain phenotype data across a multitude of environments.

The STCs would also serve to translate basic discoveries from single investigator labs worldwide into practical solutions. This could be achieved by providing matching funding to competitively funded individual/consortia labs around the world.

The example illustrated above is an ideal one and obviously many specific details need to be worked out. Regardless of the details, the main point that we need to emphasize is that we MUST ACT NOW before it is too late. In our opinion an international coordinated effort is a new paradigm to create green super crops to help solve the 9BPQ.

It is also important to emphasize that nine billion USD over a 25-year period is not an insurmountable obstacle given the problem at hand the consequences of not acting in a decisive manner. On the bright side, we as a coordinated group of plant scientists and the federal and philanthropic funding agencies can achieve a honorable and achievable goal in our lifetime that will positively affect the world as we know it, and our children's and their children's future as well.

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Index

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